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EFFECT OF ENDOCRINE DISRUPTORS ON HUMAN ENDOMETRIAL STROMAL CELLS AND THEIR INTERACTION WITH TROPHOBLAST

Scientific-disciplinary sector: BIO/09

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Abstract

Decidualization is crucial for embryo development and implantation, placenta formation and fetal growth. This process is characterized by morphological and biological changes in endometrial stromal cells that play a key role on fetal trophoblast migration and invasion. Successful placentation depends on the interaction between endometrial stromal cells and extravillous trophoblast cells. The trophoblast spheroids, a 3D culture model, is reported to appropriately mimic the in vivo situation, and reflect the cell to cell interaction. The Bisphenol A (BPA) and para-nonylphenol (*p*-NP) are endocrine disrupting chemicals (EDCs), present in the polycarbonate plastics used in many products such as food packaging, bottles and beverage cans and as an intermediate in the production of phenolic resins. Studies demonstrated that maternal exposure to EDCs, at environmentally relevant concentrations, are associated to aberrant early embryo development and uterine receptivity due to their estrogenic activity. Nowadays it is known that environmental contaminants can change stromal cell decidualization and trophoblast migration. In the present study, we developed a simple 3D culture model using transformed human endometrial stromal cells (tHESCs) and immortalized first trimester human extravillous trophoblast cells (HTR-8/SVneo). The aim of this work was to evaluate the effect of BPA and *p*-NP on endometrial stromal cells during decidualization and their interaction with trophoblast spheroids. The data showed that pre-exposition to *p*-NP of endometrial stromal cells impaired decidualization interfering on the cross-talk with trophoblast and altering lysosomes biogenesis and consequently leading to an impairment in trophoblast migration.

"You're braver than you believe,
Stronger than you seem and
Smarter than you think"

- Christopher Robin -

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1. Introduction

1.1 Materno-fetal interface

1.1.1. Human placenta

1.1.1.1. Placenta formation

Human pregnancy is an important and complex process during which the cross-talk between mother and fetus plays a key role in pregnancy development.

The placenta derives from the trophoblast, the outer layer of the *blastocyst*, a structure formed in the early development of the embryo. The embryo derives from the union of a spermatozoon with an egg cell giving rise to a new cell, called the *zygote*, the earliest manifestation of a newly formed offspring, which possesses half the genes of paternal origin and half of maternal origin. As soon as it has formed, around day 5, the zygote moves down the oviduct towards the uterus where it will go to implant. In approaching the uterus, this small being turns into a hollow sphere, blastocyst, which has an outer layer of cells that will form the placenta (trophoblast) and an inner group of cells that will become the embryo (Mayhew, 2001). Placenta development starts once that blastocyst, adheres to the uterine wall and begins the implantation process, approximately 7 days after the fertilization (Benirschke and Kaufmann, 1990).

During the formation of placenta, fetal trophoblast proliferates and differentiates forming arborescent structures named chorionic villi. In its development, the fetal trophoblast gives rise to two different cell populations: the villous (I) and the extravillous trophoblast (II). (I) The villous trophoblast constitutes the epithelial covering of the chorionic villi immersed into maternal blood (floating villi). It is distinguished in an internal layer of mononuclear proliferative cells, the villous cytotrophoblast (CT), and an external layer of multinucleated differentiated cells, the syncytiotrophoblast (ST) which, being in direct contact with the

maternal blood, allows the nourishment of the fetus and the exchange of gas (Goldman-Wohl and Yagel, 2002). In addition, the ST produces hormones such as human chorionic gonadotrophin (β-hCG) and placental lactogen (hPL), fundamental for pregnancy. In some villi, the CT cells proliferate, forming cell columns that attach the chorionic villi to the decidua (anchoring villi). This the extravillous trophoblast (EVT) which then, migrates and invades the uterine tissues to remodel the extracellular matrix (ECM) and the arterial blood vessels (spiral arteries) (**Figure 1**). The vascular changes contribute to the increase of blood flow with the aim to supply the high need of the placenta and developing fetus (Burton et al., 2009; Knöfler, 2010). Therefore, proper migration and invasion of the trophoblast are essential for the formation of the placenta and its interaction with the maternal uterus.

The placentation process is strictly regulated by molecular factors secreted by the tissues at the materno-fetal interface, the trophoblast and the decidua, and exerting their action on complementary tissues, acting as communication signals between mother and fetus (Massimiani et al., 2019). This exchange of molecules, called maternal-fetal cross-talk, includes growth factors and immunoregulatory molecules (cytokines) essential to allow efficiency and synchrony of events as well as maternal immune tolerance towards the semi-allogeneic embryo (Kelleher et al., 2018).

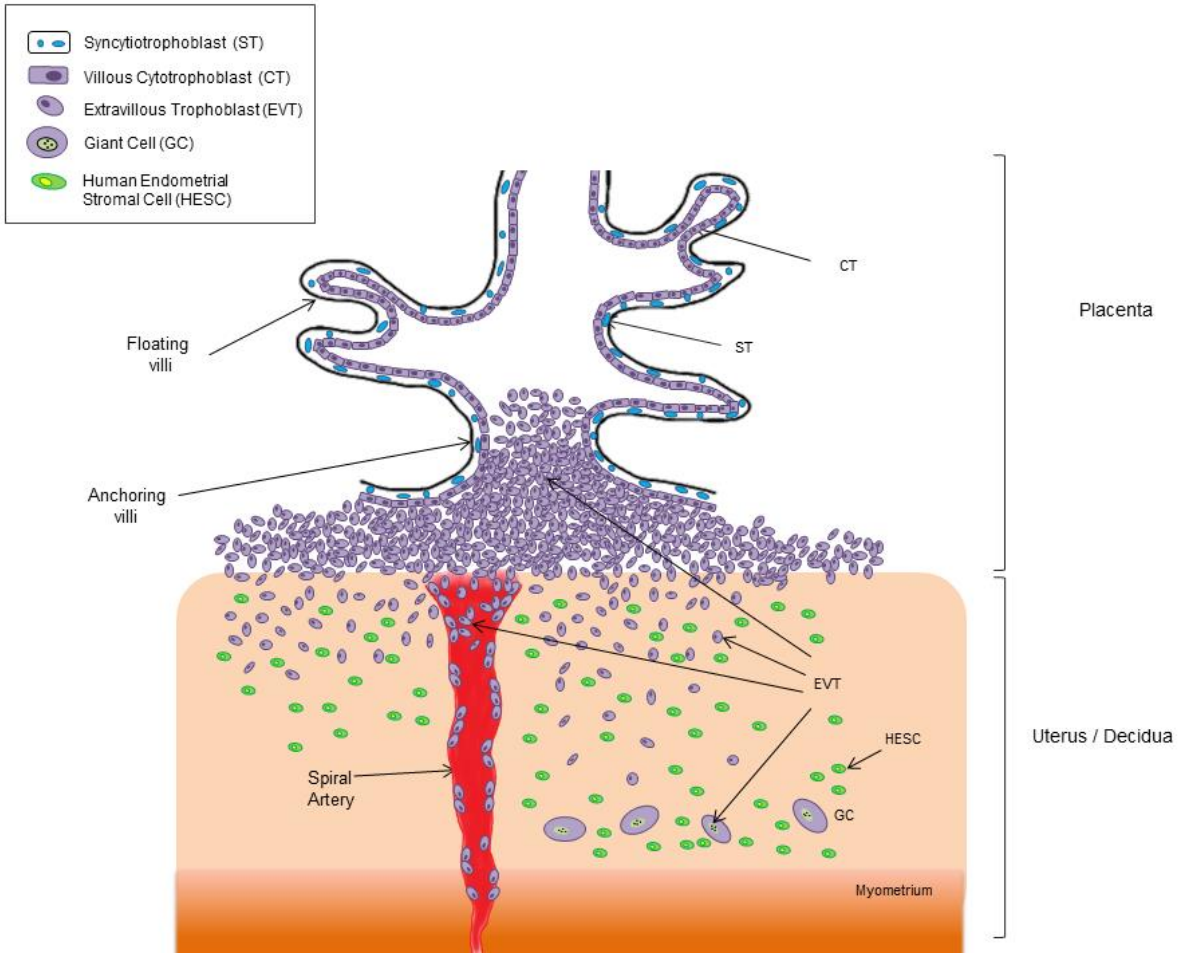

Figure 1. Trophoblast cell population in the human placenta. Chorionic villi immersed in the intervillous space (floating villi): the epithelium is formed by an internal layer of proliferative cytotrophoblast (CT) and an external layer of differentiated syncytiotrophoblast (ST). Extravillous trophoblast (EVT) deriving from the villi connected to the uterus (anchoring villi): trophoblast cell columns, invasive trophoblast in uterine tissue and spiral arteries and trophoblast giant cells (GC). Decidualized human endometrial stromal cells (HESC) can limit the EVT invasion.

1.1.1.2. Trophoblast invasion and migration

The trophoblast invasion can be characterized as a 3D spatial migration in which, EVTs perform several changes in the microenvironment. The EVT secretes matrix

metalloproteinases (MMPs), cathepsins and urokinase plasminogen activator that allow the physiological remodeling of the ECM (Cohen et al., 2006; Lala and Chakraborty, 2003; Varanou et al., 2006) Moreover, the EVT secretes proteins that are regulated by ECM receptors, such as cadherins, gap junction molecules, and integrins enabling its migration into maternal tissues and blood vessels (Staun-Ram and Shalev, 2005).

The MMP is a family of zinc-dependent proteolytic enzymes most of which are secreted as proenzymes and processed to an active form in the extracellular compartments (Nagase, 1997). The gelatinase group, known to degrade the denatured collagens (gelatines), is formed by MMP-2 (gelatinase A) and MMP-9 (gelatinase B). The interest in these enzymes is due to their capacity of degradation the type IV collagen, a major component of the basement membranes and ECM proteins, like fibronectin, laminin, elastin and vitronectin, which connect cells to the ECM (Cohen et al., 2006; Isaka et al., 2003).

During the invasion process several classes of proteins are altered. In addition to degradation of the ECM components by MMPs, the invasive trophoblast switches the adhesion molecules phenotype (Damsky et al., 1994). One of these affected proteins is the integrin, a heterodimeric membrane glycoprotein formed by α and β subunits which link ECM components to cells.

As the trophoblast differentiates into EVT, it upregulates α5β1 integrin expression, a fibronectin receptor, favoring the adhesion cell-ECM and the migration of trophoblast into the decidua (Bischof et al., 2000; Redman, 1997). Another class of altered proteins, during the invasion process, is the cell-cell adhesion molecules, more specifically, the adherent junction protein E-cadherin which is progressively downregulated as the EVT invades the maternal tissues (Zhou et al., 1997). So, the secretion of MMP and the changes in the adhesion molecules expression characterize the invasive phenotype of the EVT cell.

Moreover, as the EVT invades the decidualized endometrium it comes across with endometrial decidualized cells and immune cells. Trophoblast invasion is strongly controlled spatially and temporally by factors produced by both trophoblast and endometrial cells (Singh et al., 2011). This control takes place by different processes, such as expression of protease inhibitory molecules or by trophoblast endoreduplication processes (Graham and Lala, 1991; Martindill and Riley, 2008). The endometrial cells produce tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitors that tightly regulate the trophoblast invasion (Bischof et al., 2000; Lala and Hamilton, 1996). So, the balance between the secretion of MMPs by trophoblast and inhibition by TIMPs is a determinant factor in the control of the invasion process. As the EVT moves deeper into the decidua, its invasive capacity stops at the level of the inner third of the myometrium becoming multinucleated giant cells (GC) by an endoreduplication process (Bischof and Irminger-Finger, 2005; Martindill and Riley, 2008).

Alterations in placental development, more specifically, failure in trophoblast invasion regulation can lead to gestation complications, such as early pregnancy loss (Ball et al., 2006), fetal growth restriction (Khong et al., 1986; Romero et al., 2011), preeclampsia (Goldman-Wohl and Yagel, 2002) and placenta accreta (Li et al., 2014). In a preeclampsia situation, alteration on trophoblast differentiation limits its invasion in the decidua providing a poor remodeling of the blood vessels (Fisher, 2015). Otherwise, in the placenta accreta disorder, an excessive invasion of EVTs into the uterus leads these cells to reach the underlying uterine myometrium, the uterine serosa or in some situations extending to pelvic organs (Li et al., 2014; Piñas Carrillo and Chandraharan, 2019).

1.1.2. Endometrium

The intimate contact between the mother and the fetus is coordinated in order to ensure a successful pregnancy. The maternal uterus consists of various cell types including the epithelial cells which line the uterine and the glandular lumen, the stromal and endothelial cells. The human endometrium undergoes, monthly, structural and physiological changes controlled by steroid hormones, 17-β Estradiol (E2) and progesterone (P4) (Schaefer et al., 2010; Sharkey and Smith, 2003). In the proliferative phase, E2 promotes mitosis in several types of uterine cells including stromal fibroblasts, vascular and epithelium cells to repair the endometrial tissue released during the menstruation period. On the secretory phase, P4 induces changes in the epithelial cells favoring adhesion and implantation of the blastocyst (Hombach-Klonisch et al., 2005) and in the stromal cells inducing their differentiation into decidual cells (decidualization) preparing the uterine mucosa for embryo implantation (Hawkins and Matzuk, 2008) (**Figure 2**).

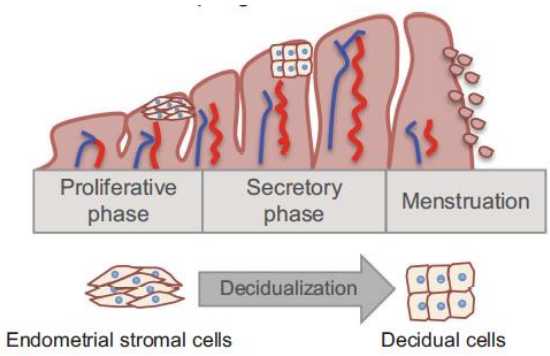

Figure 2. Morphologic changes of endometrial tissue during the menstruation cycle. (Adapted from Devis-Jauregui et al 2021).

1.1.2.1. Decidualization

Endometrium is a dynamic tissue in which cyclic growth and breakdown occur monthly. Decidualization of the endometrium occurs spontaneously in every normal menstrual cycle during the late secretory phase, when the uterus is receptive to embryo attachment, in the event that fertilization of the egg has occurred (Pellicer et al., 2002) (**Figure 2**). If blastocysts implants the decidualization intensifies for the maintenance of high levels of P4 (Brosens et al., 2002).

During decidualization, including extracellular matrix remodeling and becomes more vascularized, reaching a maximum at around days 21-23, a period named "implantation window" (Donaghay and Lessey, 2007; Yoshinaga, 1988). This is the time of maximum uterine receptivity, a critical event in female reproduction due to its limited duration and occurrence only once per cycle. In this period, the apical uterine epithelium surface develops pinopodes (micro protrusions) and the endometrial glands enhance their secretory activity, creating a suitable nutritive *milieu* for the embryo adhesion and implantation (Caballero-Campo et al., 2002; Gellersen and Brosens, 2014). The elongated fibroblast-like human endometrial stromal cells (HESCs) differentiate into decidual cells in response to progesterone, characterized by morphological changes and biochemical modifications. These changes consist in accumulation of lipids and glycogen in the cytoplasm, increase and dilatation of rough endoplasmic reticulum (ER) and Golgi apparatus, increased synthesis of extracellular matrix proteins such as type IV collagen, fibronectin and laminin as well as the secretion of prolactin (PRL) and insulin-like growth factor binding protein-1 (IGFBP-1) (Christian et al., 2002; Dunn et al., 2003; Gellersen and Brosens, 2014; Pan-Castillo et al., 2018). Being the PRL and IGFB-1 the main products of endometrial stromal cells, these two

molecules are used as key markers of the decidualization process (Gellersen and Brosens, 2014).

Furthermore, the decidua is able to regulate fetal trophoblast invasion establishing with it an intense cross-talk via molecular and cellular interaction (Staun-Ram and Shalev, 2005). The ECM consists of a heterogeneous network of collagen and fibronectin that supports EVT migration by concerning interactions with cell surface integrins, α1β1 and α5β1 respectively (Lopez-Mejia et al., 2013). Furthermore, decidualized human endometrial stromal cells (HESCs) express a tissue specific variant of fibronectin, which being one of the ECM components can promote trophoblast invasion (Talukder et al., 2018). So, during decidualization the decidua is able to control the invasion of EVT by up- or down-regulating expression of the proteins present in the ECM.

1.1.2.2. Lysosomes and decidualization

It has been reported that lysosomes participate in many physiological processes including implantation and placentation (Nakashima et al., 2020). Changes in decidualized stromal cells morphology besides the accumulation of lipids and glycogen include an increase of the number of lysosomes in their cytoplasm (Kajihara et al., 2014). Lysosome activity has been associated with endometrial health and preimplantation of embryo. Lysosomal dysfunction showed effects on preimplantation process. Downregulation of lysosome-associated membrane protein 1 and 2 (LAMP-1 and LAMP-2) leads to mouse embryo arrest (TSUKAMOTO et al., 2013).

Lysosomes are acidic and membrane-bound organelles that participate in many physiological processes such as endocytosis/phagocytosis, autophagy and exocytosis

(Eskelinen et al., 2003) (**Figure 3**). Lysosomes are originated by the Golgi compartment as small vesicles that appear extremely electron dense and are named primary lysosomes. These vesicles can fuse with other vesicles and vacuoles inside the cell to form the secondary lysosomes (Samie and Xu, 2014). It is a level of secondary lysosomes that we have the degradation and recycling of cellular material. The particles destined for degradation come from extracellular compartment by invagination of the plasma membrane forming endocytic vesicles that fuse with lysosomes giving rise to endolysosome enabling the particles degradation (Huotari and Helenius, 2011). Autophagy is another way in which intracellular molecules are addressed to lysosomes to de degraded. The autophagosome englobes cytoplasm organelles and proteins, fuses with lysosome forming the autolysosomes, broke down the autophagic cargo and releases the monosaccharides, amino acids and fatty acids into the cytoplasm making them available to be reused (Bildirici et al., 2012; Ruivo et al., 2009).

Lysosomes are also subjected to lysosomal exocytosis (Medina et al., 2011). The acidic organelles are targeted to the plasma membrane where they fuse and release their content rich in acid hydrolases outside the cells (Chieregatti and Meldolesi, 2005). Lysosomal exocytosis plays an important role in several cellular processes, such as plasma membrane repair, cell signaling and immune responses (Andrews, 2002; Bossi and Griffiths, 2005; Rodríguez et al., 1997).

It is well known that the action of proteases plays a key role during decidua remodeling regulating the invasion and implantation processes (Walter and Schönkypl, 2006). Besides the activity of the MMPs, lysosomal proteases such as the Cathepsins (CTPS D) which are active in an acidic environment and are released outside the cells by lysosomal exocytosis,

are able to digest matrix proteins, including fibronectin and type IV collagen (Buck et al., 1992) and to activate other proteases that play a role in matrix degradation (Conus and Simon, 2008; Vizovišek et al., 2019).

In synthesis, decidualization is essential for successful blastocyst implantation facilitating its interaction with the uterine epithelium, its invasion into the maternal uterus and creating an ideal nest for fetal growth and development (Caballero-Campo et al., 2002; Ghosh and Sengupta, 1998; Pellicer et al., 2002). Any alteration during decidualization process could to embryo implantation and placentation failure, miscarriage and intrauterine fetal growth restriction (Cha et al., 2012; Gellersen et al., 2007).

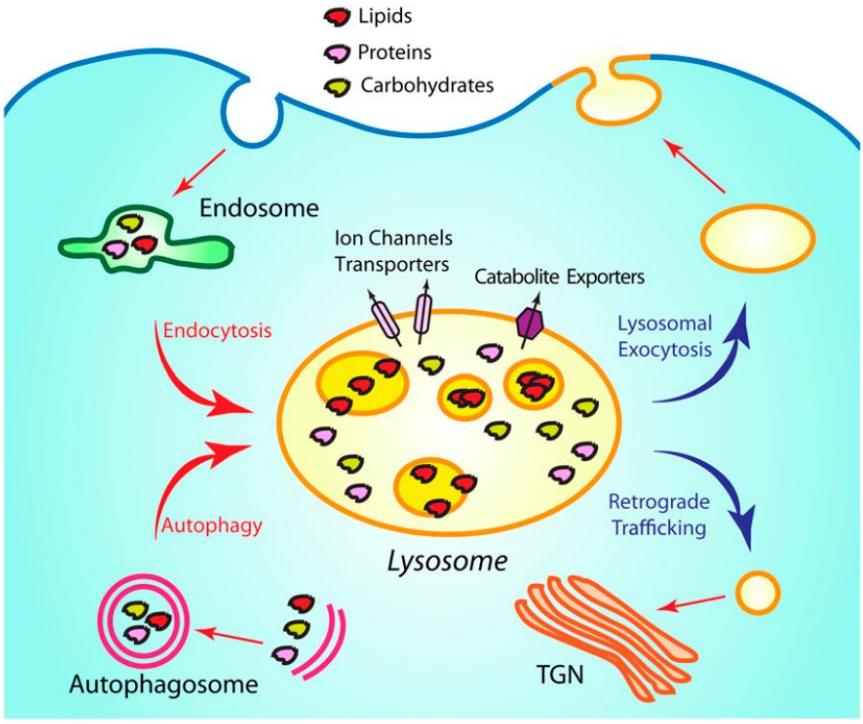

Figure 3. Lysosomal membrane trafficking pathways. (Adapted from Samie and Xu, 2014).

1.2. *In vitro* models

The placenta is a very complex organ with a relevant ability to establish an intense and refined cross-talk between both, the uterus and the fetus. The placenta is a species-specific organ, showing different features between one animal species and another. On the basis of the relationship between this organ and the uterine tissues, the placenta takes different names, in different species. It is called *hemochorial* when the trophoblast invades the uterine tissue up the spiral vessels and makes direct contact with the maternal blood, *endothelio-chorial*, when the trophoblast reaches the endothelium of the uterine vessels but without destroying it and *epithelio-corial* when the trophoblast is affixed to the uterine epithelium without penetrating it. Human placenta is of hemochorial-type like that of mice and rats, although presenting anatomical and functional differences with these species (Rai and Cross, 2014; Soares et al., 2018). Because of the numerous variations among the different types of placenta, studies in humans are mainly performed in tissues from human pregnancy. Over the years several *in vitro* models have been proposed to facilitate the study of the maternal and fetal compartments. In particular, human placentation research has been mainly based on the use of 2D and 3D models of trophoblast and/or endometrial cells.

Primary trophoblast cells can be obtained by enzymatic digestion from fresh placenta. These cells, although well representing the *in vivo* situation, have low proliferative activity and cannot be cultured for long periods, which makes it difficult to perform some type of investigation. So, many studies have used cell lines that can proliferate indefinitely such as choriocarcinoma-derived cells or immortalized cell lines. Many studies have used BeWo, JEG-3 and JAR cells (choriocarcinoma-derived cells) due to their ability to produce hormones, including hPL, β-hCG and P4, and to differentiate in syncytiotrophoblast

(Almeida et al., 2021; Serrano et al., 2007). On the other hand, HTR-8/SVneo (immortalized cell line) is considered to be representative of EVT based on its invasive features which expand out of the chorionic villi and migrate into the maternal decidua (Graham et al., 1993). HTR-8/SVneo cells are originated from first trimester human placenta and immortalized by transfection with simian virus 40 large T antigen (Graham et al., 1993).

Therefore trophoblast monolayers have been used to investigate placental mechanisms, several studies have approached 3D sphere-like tightly bound cellular aggregates, to study human implantation. The 3D-spheroids culture model represents more realistically the *in vivo* process implantation respect to 2D monolayers culture, once trophoblast spheroid mimics the blastocyst at the early phase of attachment and invasion to the maternal decidua (Hohn and Denker, 2002; Wang et al., 2013).

There is a diversity of processes to obtain the 3D spheroids model. It is generally obtained by the cells culture in low-attachment plates, or plates covered with non-adhesive materials, for example, agarose, hyaluronic acid (HA) (Basharat et al., 2020), as well as hanging drops (Jensen and Teng, 2020; Tiwari et al., 2004) In these methods the cell attachment is inhibited by different ways, allowing spontaneous formation of spheroids by promoting cell–cell adhesion (Achilli et al., 2012; Ryu et al., 2019). Costa and co-workers, elucidated the spheroid organization demonstrating that it is formed by three layers: a. external layer formed by proliferative cells; b. intermediate layer composed by quiescence cells; c. inner layer which has acidic and hypoxic characteristics constituted by necrotic cells (Costa et al., 2016).

The HESCs are representative cells of maternal decidua, which can be isolated from endometrial human biopsies (Menkhorst et al., 2012; Pan-Castillo et al., 2018) and cultured as a primary cells. Given the difficulty of maintenance of primary culture, these cells can be

immortalized by telomerase transfection facilitating it culture (tHESC) (Krikun et al., 2004). These cells preserve the characteristics of the endometrium tissue making it an ideal model to *in vitro* studies.

Over the years several *in vitro* models have been developed including trophoblast and endometrial cell cultures. Moreover, in order to better reflect the *in vivo* situation, 3-dimensional models of human trophoblast and co-culture systems including both fetal and maternal uterine cells have been developed. A number of studies have done a variety of 3D co-culture models (Fan et al., 2020a; Gao et al., 2019; Li et al., 2018; Moser et al., 2010) including fetal trophoblast spheroids or placenta explants in direct or indirect contact with endometrial stromal cells. The 3D co-culture with direct contact in some studies was performed with trophoblast spheroids on a HESC monolayer (Gao et al., 2019; Wongwananuruk et al., 2016) or with HESCs embedded in a matrix (Haider et al., 2018; Wang et al., 2012). The co-culture with an indirect contact was done with trophoblast treated with HESC-conditioned medium (Mannelli et al., 2014; Menkhorst et al., 2019) or using a Transwell culture with trophoblast on the upper chamber and the HESCs on the lower chamber (Fan et al., 2020b; Manzan-Martins and Paulesu, 2021; Wongwananuruk et al., 2016) (**Figure 4**).

Therefore the 3D co-culture models showed to be an interesting tool for the materno-fetal studies, due to its potential to represent better the *in vivo* situation.

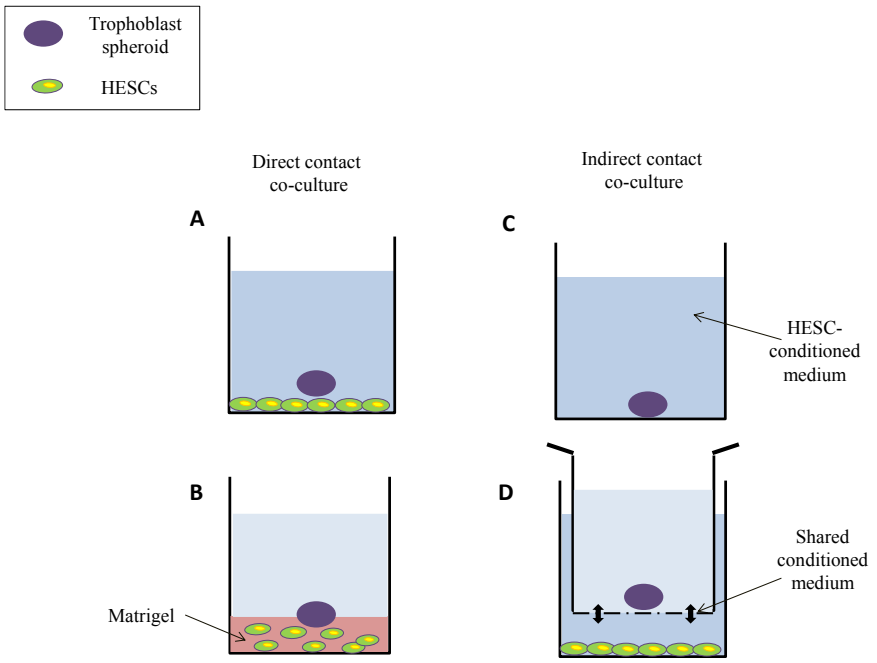

Figure 4. Co-culture models of fetal trophoblast and human endometrial stromal cells. Trophoblast spheroids on HESC monolayer (A) or HESCs in a matrix (B), trophoblast spheroids in HESC-conditioned medium (C) or in a Transwell with trophoblast spheroids in the upper chamber and HESCs in the lower chamber (D) (Modified by Manzan-Martins and Paulesu, 2021).

1.3. Endocrine Disruptor Chemicals

The Endocrine Disruptor Chemicals (EDCs) constitute a large and heterogeneous group of substances including environmental contaminants, compounds used in industrial and consumer products and natural compounds as phytoestrogens (Caserta et al., 2008). Many of EDCs possess chemical-physical characteristics that allow their persistence in the environment for long periods and the accumulation in the fat and in other tissues of animals, and humans, that assume these compounds through the food chain (Galloway et al., 2010).

Given the widespread distribution of the EDCs in the environment, it is almost inevitable for humans to avoid their contamination. EDCs concentrations have been indeed found in

tissues and body fluids of most inhabitants even in agricultural and non-industrialized areas (Ghisari and Bonefeld-Jorgensen, 2009; Ho et al., 2017). Many of EDCs share many analogies with the natural estrogens and can interfere with the role of these hormones in reproductive processes. These chemicals in fact are able to act on estrogen-responsive organs by binding to estrogen receptors isoforms, ERα and ERβ, and regulating target gene expression (Baker and Chandsawangbhuwana, 2012; Nagel and Bromfield, 2013). In general, the effect of different EDCs with estrogen-like activity have been shown in the brain, in the thyroid, as well as in female and male reproductive tissues (Caserta et al., 2008; Ho et al., 2017).

In the last years has been an increasingly consideration of the impact of EDCs on prenatal exposure. Prenatal life is a critical and plastic period of development during which external insults can lead to permanent changes in cells, tissues and even in the whole organs (Barker et al., 2002). On the "Fetal origin of adult diseases" the hypothesis postulated by Barker consist on any alteration in growth and development during early life can persist even after birth, and lead to an increased risk of developing dysfunctions in adulthood (Barker, 1990; Barker et al., 2002). Therefore, exposure to EDCs during early development can lead to some placental disorders and may also predispose the fetus to the risks for developing chronic diseases in adult life (Benincasa et al., 2020; Burton et al., 2016).

Among the adult disorders that can be potentially caused by the prenatal exposure to EDCs are neurological, cardiovascular and metabolic diseases (Bronson and Bale, 2016) .

1.3.1. Bisphenol A

Bisphenol A (BPA), a representative EDCs with well recognized estrogenic activity and wide diffusion in several items to produce plastics and resins. BPA is a monomer that can be found in polycarbonate plastics and metal cans used for food and beverages, baby toys, medical and dental devices, and many other things with which we are in straightly contact every day (Berger et al., 2010; Ghisari and Bonefeld-Jorgensen, 2009; Ikezuki et al., 2002). Although the use of BPA in specific products for babies has been banned in some countries of the European Union (European Union (EU), 2011) in the last years, this chemical is present in such a large number of items that is almost impossible to avoid contamination from this product.

Measurable levels of BPA have been indeed detected in fluids and tissues of the majority of individuals (Calafat et al., 2005; Rubin, 2011). Human prenatal exposure to BPA is revealed by the detection of this chemical in the placenta amniotic and follicular fluid, as well as in cord blood (Cao et al., 2012; Ikezuki et al., 2002). BPA can also be transferred through the placenta and reach the fetus (Mørck et al., 2010). Besides its estrogenic activity, BPA can also exhibit antiprogestin activity, binding to progesterone receptors (Baker and Chandsawangbhuwana, 2012; Scippo et al., 2004). Estrogens and progesterone are key hormones for reproduction and pregnancy, interference with the action of these hormones by BPA can agonize or antagonize their effect by altering normal physiologic processes (Lassen et al., 2014; Leonel et al., 2020; Muñoz-de-Toro et al., 2005).

Many studies have been performed to test the effects of BPA in the prenatal life in order to evaluate the potential risks of maternal exposure to these chemicals on fetal growth and development (Benincasa et al., 2020; Ermini et al., 2021; Strakovsky and Schantz, 2018).

Furthermore, BPA can cross the placenta and interfere in implantation and placentation processes, leading alterations on migrations and invasion of trophoblast cells (Wang et al., 2015; Wei et al., 2020). In that way, our group have elucidated by many works the interference of BPA on the placenta. Mannelli and co-workers showed that BPA affect decidualization markers, such as a decrease on PRL expression compromising the uterine receptivity (Mannelli et al., 2015). Furthermore, BPA also interferes with the differentiation of trophoblast, increasing the endoreduplication processes in HTR-8/SVneo, consequently reducing the trophoblast invasion rate (Spagnoletti et al., 2015). This chemical also lead to an increase of β-hCG secretion and cell fusion BeWo cells, a characteristic of the ST phenotype (Narciso et al., 2019).

Therefore the presence of BPA at the materno-fetal interface may have a significant effect on placentation and pregnancy development.

1.3.2. *para*-Nonylphenol

para-Nonylphenol (*p*-NP) is another potential hazardous chemical representative of EDCs. *p*-NP is an alkylphenol ethoxylate mostly used as an intermediate in the production of phenolic resins, such as household and cleaning products personal care products, chemical stabilizers, pesticides and an antioxidant used widely in some plastics (de Weert et al., 2008; White et al., 1994). These alkylphenol ethoxylates are sprayed into rivers and sea with urban and industrial wastewater, and once into the environment, they are broken down by microorganisms into nonylphenol ethoxylate by-product (Rudel et al., 2003; White et al., 1994). *p*-NP can be ingested by human via contaminated food or water, by inhalation or cutaneous absorption (Guenther et al., 2002; Monteiro-Riviere et al., 2000). This compound

was synthesized in 1940 and widely used until 2003 when the European Union implemented a reduction policy of its use for the industry (Commission Directive 2003/53/EC, 2003). Although nowadays its use has been greatly reduced, *p*-NP is still detected in several environmental matrices such as water and soil (David et al., 2009; Gatidou et al., 2010).

Moreover, studies have reported that *p*-NP exhibits estrogenic activities *in vitro* (Soto et al., 1991; White et al., 1994) and *in vivo* (Laws et al., 2000) experiments. In 1991, Soto and co-workers showed that *p*-NP induces estrogen and progesterone receptor synthesis and cell proliferation in breast cancer cells (Soto et al., 1991). Furthermore, there are evidence that *p*-NP can cross the placental barrier and induce calbindin-D9k (CaBP-9k) mRNA and protein expression in the neonatal uterus (Hong et al., 2004; Kwack et al., 2002). Besides that, *p*-NP has been detected in blood samples of pregnant women and in fetal serum at a concentration of 5.68 ng/g and 2.95 ng/g, respectively (Cao et al., 2012; Chen et al., 2008; Li et al., 2014; Schönfelder et al., 2002).

In the last decade, besides the research about the effects of BPA, our group has been dedicated to studying the maternal exposure to *p*-NP. In particular, the treatment of placenta explants with low doses of *p*-NP showed an increase in β-hCG secretion and cell apoptosis (Bechi et al., 2006). Beyond that, the exposure of BeWo cells to *p*-NP demonstrated that the release of β-hCG was altered resulting in hormetic or biphasic behavior, in which high concentrations of *p*-NP decrease the β-hCG secretion and lower concentrations increase the hormone secretion (Bechi et al., 2013). The potential of *p*-NP as well as of other EDCs in altering β-hCG production suggests that this action could exert pregnancy pathologies, in that way β-hCG serum levels could represent a potentially useful clinical biomarker of inappropriate prenatal exposure to EDCs (Paulesu et al., 2018). We have shown that *p*-NP

can also induce the release of placental cytokine secretion, such as GM-CSF and IL-10 (Bechi et al., 2010). Furthermore, treatment with low doses of *p*-NP reduced trophoblast migration and invasion on HTR-8/SVneo cells by increasing the endoreduplication process and reduced trophoblast/endothelial interaction (Spagnoletti et al., 2015).

Therefore, based on the extensive studies carried out by our group on BPA and *p*-NP and the relevance of these compounds during pregnancy we decided to elucidate the role of these chemicals on trophoblast interaction with endometrial stromal cells.

2. Aim of the study

This study aimed to examine the effect of EDCs pre-natal exposure in tissues at the materno-fetal interface. In particular, we investigated if the maternal contamination with BPA or *p*-NP was able to impair stromal cell decidualization and placentation. In this context, a 3D co-culture model was used to represent the complex interaction between maternal and fetal tissues.

3. Material and Methods

3.1. Cell culture

3.1.1. Culture of Endometrial Stromal (tHESCs) and HTR-8/SVneo cells

Human endometrial stromal cell line (tHESC; ATCC® CRL 4003™, Manassas, VA, USA) and the HTR-8/SVneo cells (a kind gift from Professor Charles Graham of the Department of Anatomy and Cell Biology at Queen's University (Kingston, ON, Canada) were used in this research project. tHESC and HTR8 were cultured in DMEM-F12 medium without phenol red (Sigma Chemical Co). supplemented with 10%(v/v) fetal bovine serum (FBS) (Biochrom, Berlin,Germany), 100U/ml penicillin/streptomycin, and 1% (v/v) glutamine (2mM; Sigma Chemical Co), named as complete medium. 1% (v/v) sodium pyruvate (100 mM; Pan Biotech) was added in tHESCs medium. The cells were maintained in culture in 75 cm2 flasks in a humidified air atmosphere (5% CO_2) at 37°C until 80% confluence reached.

3.1.2. HTR-8/SVneo Spheroids

HTR-8/SVneo spheroids (SPHD) were obtained as described by Friedrich (Friedrich et al., 2009). Briefly, agarose-coated plates. were prepared using agarose (SeaKem® LE Agarose, Cambrex) dissolved in deionized autoclaved water at a concentration of 1,5% . 96-well plate was coated with 100 µl/well of agarose solution. After solidification, the agarose formed a concave low attachment surface. To ensure sterilization, the agarose-coated plate was exposed uncovered to the UV-light of the laminar flow hood for 30 min.

HTR-8/SVneo cells were seeded in 25 cm^2 flasks and cultured until 70% confluency. The cells were then trypsinized counted and plated (1000 cells in 100 µl) in agarose-coated wells.

After that, cells were maintained in culture in a humidified air atmosphere (5% CO_2) at 37°C until the formation of SPHDs (48h).

3.2. Experimental Plan

3.2.1. *in vitro* decidualization and conditioned medium from tHESC exposed to treatment with BPA and *p*-NP

tHESCs (1.5×10^5) were cultured in 6 well-plates with steroid hormones to simulate the *in vivo* conditions. The cells were first maintained for 78 hrs without hormones. The medium was then replaced with a fresh one containing E2 (10-8 M) (Sigma–Aldrich) and 2%(v/v) to better replicate the proliferative phase (priming) and FBS without E2 (charcoled FBS). Finally, for the next 78 hrs the cells were exposed to E2 (10 nM) + P4 (1 mM) + cAMP (0,5 mM) (Sigma–Aldrich) to mimic the secretory phase. In the treated group, BPA (1 nM) (Sigma–Aldrich) or *p*-NP (1 nM and 1 pM) (Sigma-Aldrich) was added to the medium containing the hormone cocktails that simulate the secretory phase. The E2+P4 were added to the medium every day, meanwhile the cAMP and the treatments (BPA or *p*-NP) were added every two days, when the medium was renewed (**Figure 5**). In parallel cultures, treatment with BPA or *p*-NP was substituted with the same aliquot of ethanol 0.1% (vehicle), the solvent in which BPA or *p*-NP were dissolved (control cultures).

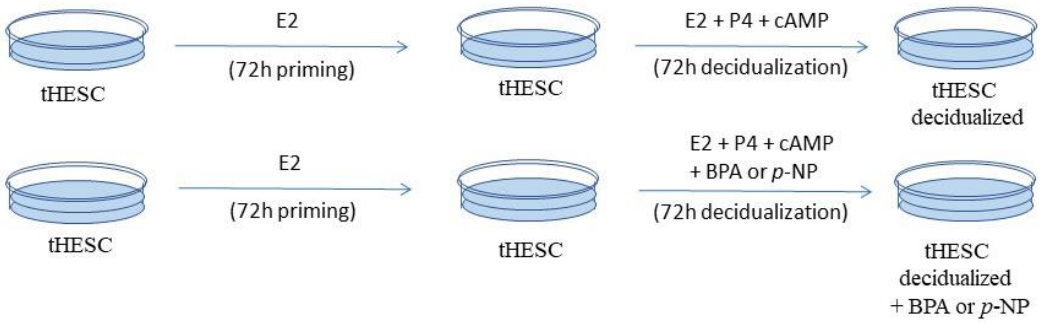

Figure 5. tHESC Decidualization and BPA or *p*-NP treatment.

At the end of 72 h of incubation, the conditioned medium (tHESC-CM) was collected, centrifuged at 13000 g, at 4 °C for 10 min and stored at -20 °C for futures analysis (see below).

The cells used for qPCR analysis were harvested in TriReagent solution (Roche), whereas those for protein analysis were extracted in M-PER lysis buffer (Mammalian Protein Extraction Reagent) supplemented with 1%(v/v) of HaltTM Protease and Phosphatase Inhibitor Single-Use cocktail (Thermo Fisher). Both of them were sonicated on ice, centrifuged at 12000 g for 10 min at 4 °C and stored at -80 °C until qPCR and western blotting analysis.

3.2.2. Trophoblast spheroids in 3D co-culture model with decidualized tHESCs

The 3D co-culture model involves the decidualization of tHESC monolayers (described previous at 3.2.1 item), the SPHD formation (described above at 3.1.2 item) and their 3D co-culture model.

In order to verify the growth and the localization of the SPHD in the co-culture, HTR8 cells were labelled. Briefly, HTR-8/SVneo cells were seeded in 25 cm^2 flasks, cultured until 70% confluency and stained with 10 mM CellTracker Green CMFDA (5-chloromethylfluorescein

diacetate; Molecular Probes, Life Technologies) for 30 min at 37°C in the dark. The cells were then trypsinized and 1000 cells in 100 µl were plated on agarose-coated plates as described above (see 3.1.2 item) and cultured for 48 h (**Figure 6**).

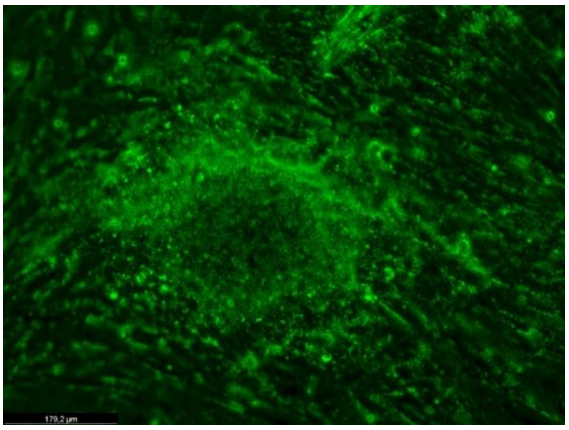

Figure 6. Fluorescent stained spheroid (Sccale bar = 179,2um).

The stained HTR-8/SVneo SPHDs, were carefully transferred onto the decidualized tHESC monolayer previously exposed to BPA or *p*-NP, and cultured with complete DMEM-F12 medium without phenol red and with 2%(v/v) charcoled FBS. The co-culture was maintained in a humidified 5% CO_2–95% air atmosphere at 37°C, for 48h (**Figure 7**).

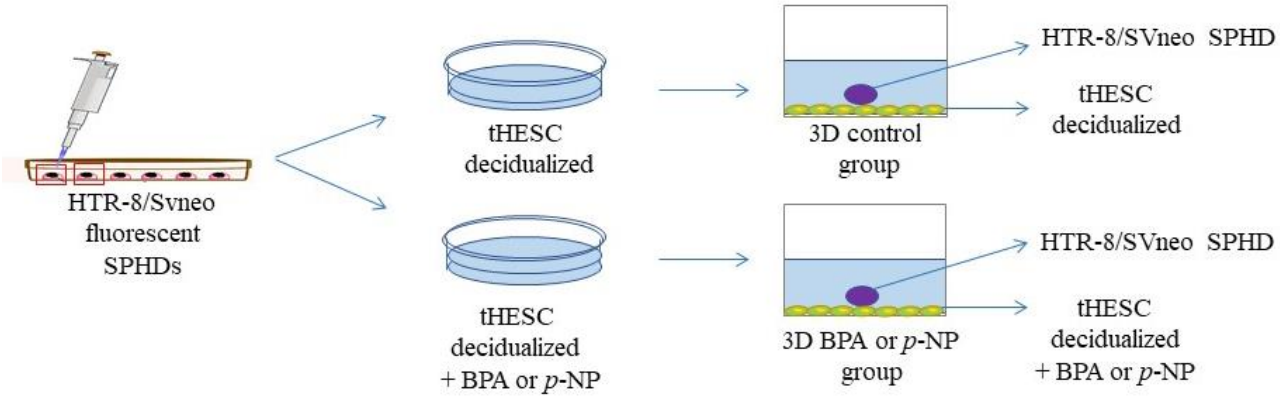

Figure 7. Workflow of 3D co-culture model. The labelled spheroids are seeded onto the decidualized tHESC monolayer previously exposed to a BPA, *p*-NP or only vehicle.

3.2.3. Trophoblast spheroid outgrowth

Pictures were taken after 24h and 48h of co-culture to measure the size of the SPHD in both control and treated (BPA or *p*-NP) groups (**Figure 8**). The pictures were captured using a fluorescent microscopy (Leica DMI 4000 B). The areas were measured by ImageJ software ("ImageJ," n.d.) (NIH Image; https://imagej.nih.gov/ij/) and the relative outgrowth area was calculate using the following formula:

$$\text{SPHD outgrowth} = \frac{\text{48h outgrowth area}}{\text{24 h outgrowth area}} . 100$$

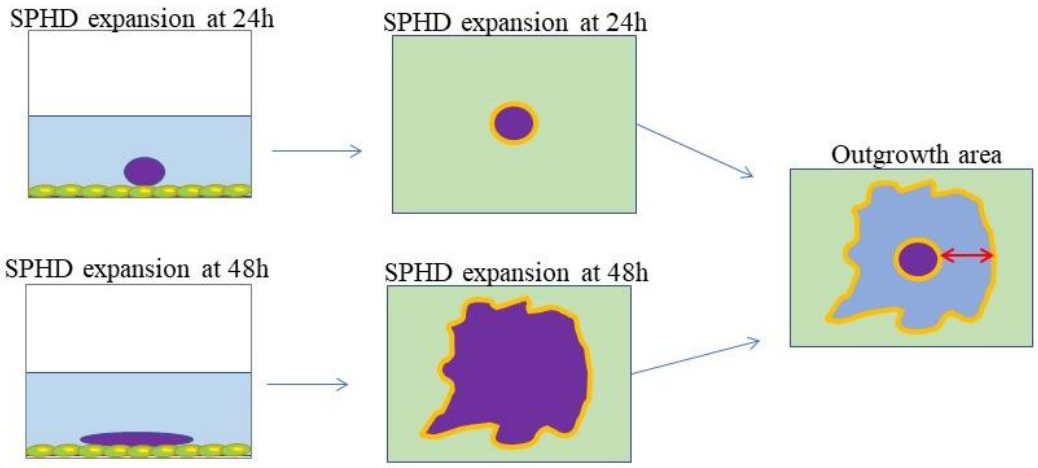

Figure 8. Growth of spheroids on the tHESC monolayer and representative scheme of outgrowth area.

3.2.4. Scratch test in HTR-8/SVneo cells with tHESC conditioned media pre-exposed to *p*-NP.

In order to examine cell migration, the HTR-8/SVneo (2.5 x 10^5/well) were seeded in 12 well-plate and cultured till 80% confluency. A straight scratch using a plastic tip was

performed on the monolayer cells and the medium was replaced with complete DMEM medium containing 2% FBS diluted with 1:1 with the tHESC conditioned media (tHESC-CM) pre exposed to *p*-NP (1 nM and 1 pM) or only to vehicle and cultured in controlled conditions, for 18 h. The images were taken with a phase-contrast light microscope at 0 h and 18 h after scratching.

At the end of 18 h, the cells were extracted as described above (see item 3.2.1) and stored at -80 °C until protein analysis.

3.3. Biochemical and Histological analysis

3.3.1. SDS Page and Western Blotting

3.3.1.1. Sodium dodecyl sulphate electrophoresis on polyacrylamide based gel (SDS PAGE)

In order to remove the salt excess of the conditioned media, it was performed a solvent-based precipitation (acetone and methanol). 200 µl of conditioned media were supplemented with 2 mL of cold acetone/methanol (1:1) and incubated 24 h at -20°C. After that, the solution was centrifugated at 13000 g for 15 min at 4°C and the pellet resuspended in 500 µl of cold acetone and incubated again at -20°C for 2 h. followed by another centrifugation at the same conditions. The obtained pellet was resuspended and directly loaded on the polyacrylamide gel.

Protein concentration of samples was determined by Bradford Protein Assay (Bio-Rad). 30µg of total protein were loaded on 7% and 10% acrylamide/bis-acrylamide electrophoresis gel and separated as describe by Laemmli (Laemmli, 1970) using constant

voltage (135V for 75 minutes) and a TGS buffer (25mM Tris, 190mM glycine, 0.1%(w/v) SDS).

3.3.1.2. Western Blotting

Proteins were electro-transferred onto nitrocellulose membranes for 2h at constant voltage (100 V). The membranes were incubated for 1 h at room temperature in 5%(w/v) non-fat dry milk in Tris-buffered saline pH 7.2 (TBS) as a blocking solution. After that, membranes were incubated overnight at 4°C with the following primary antibodies described at the **Table 1**. The nitrocellulose membranes were then washed three times with TBS-Tween20 (0.1% Tween20 in TBS) and incubated with the appropriated horseradish peroxidase-conjugated IgG (Bio-Rad) at dilution of 1:2000 for 1 hour at room temperature. Another three washes with TBS-Tween20 was done.

The reaction was revealed using chemiluminescent reagents (BioRad) and digitalized with CHEMIDOC Quantity One 1D Analysis Software and quantified with.

Table 1. Antibody used on Western Blot analysis.

Antibody	Biological Origin	Dilution	Product by
anti-human LAMP1	mouse	1:200	Santa Cruz
anti-human CD9	rabbit	1 : 250	Sigma–Aldrich
anti-human PRL	mouse	1 : 500	Santa Cruz
anti-human Fibronectin	rabbit	1 : 500	Sigma–Aldrich

anti-human α5 Integrin	mouse	1 : 500	Santa Cruz
anti-human CX-43	rabbit	1 : 1000	Cell Signaling
anti-human MMP-2	rabbit	1 : 1000	Abcam
anti-human MMP-9	rabbit	1 : 1000	Abcam
anti-human CTPS D	rabbit	1 : 1000	Cell Signaling
anti-human GAPDH	mouse	1 : 2000	Sigma–Aldrich
anti-human β-actin	mouse	1 : 3000	Cell Signaling

3.3.2. Quantitative Real Time PCR (qPCR)

Cells were removed in TriReagent solution and total RNA was purified with Direct-zol RNA MicroPrep Kits (Zimo Research) following the manufacturer's instructions. RNA concentration and quality was determined spectrophotometrically by Nanodrop. The absorbance ratio (260nm and 280nm) was approximately 2. After that, 1μg RNA was used to synthesize cDNA by iScript Adv cDNA kit for RT-qPCR (Bio-Rad) according to the manufacturer's recommendations and the cDNA was then, stored at -20°C. Primers used for gene amplification are PRL and TFEB. Quantitative real time PCR was performed on Applied Biosystems 7900HT Fast-RealTime System (Applied Biosystems) with Ss0Advanced Universal SYBR® Green Supermix (Bio-Rad) according to the manufacturer's instructions. The primerPCR cycling protocol for amplification process consists of a initial activation cycle of 2 min at 95°C, a denaturation with 40 cycles (5 sec at 95°C) and 1 cycle of both annealing and extension (30 sec at 60°C). The melt curves were obtained by an increment (0.5°C) in temperature from 65-95°C (5 sec/step). The expression levels of mRNA

were normalized to that of GAPDH and calculated using the 2−△△Ct method (Livak and Schmittgen, 2001).

3.3.3. Phosphatase acid assay

In order to measure the acid phosphatase activity on tHESC conditioned media, Alkaline Phosphatase Yellow (pNPP) Liquid Subtrate System ((Sigma–Aldrich) was used. 20 µl of samples and 180 µl of substrate solution were deposited in wells of a 96 well-plate. The plate was incubated for 1 h at 37°C. Thereafter, the reaction was stopped by adding 100 µl of NaOH (1M). The reaction was revealed measuring the absorbance through a microplate reader in the range 405-450 nm.

3.3.4. Immunoflorescence

8.5 x 10^4 tHESCs were seeded on rounded slides in a 12-well plates and decidualized as described above (see 2.2.1 item). After 72 h of decidualization, the slides were washed, fixed with cold formaldehyde for 10 min and permeabilized with 0.2% Triton for 5 min at room temperature. The slides were blocked in 5% Bovine Serum Albumin (BSA) for 30 minutes and incubated overnight with mouse anti-human LAMP-1 (Santa Cruz) diluted 1 : 250 in PBS. The day after, the slides were washed and incubated for 1 h with goat anti-mouse IgG (H&L), DyLightTM 488 (ThermoFisher) diluted 1:300 in PBS. Images were acquired on Zeiss LSM700 confocal microscope and analyzed using Image J software (NIH Image; https://imagej.nih.gov/ij/).

3.4. Statistical analysis

Densitometries were performed using ImageJ software (NIH Image; https://imagej.nih.gov/ij/). Data are represented as mean ± SE and analyzed with GraphPad Prism 8.3.0 (GraphPad Software, Inc., San Diego, CA). The obtained data were statistically examined through the 'One-way Anova test', followed by 'Tukey's multiple comparison test'. $p < 0.05$ was considered to be statistically significant.

4. Results

4.1. Effect of BPA on spheroid expansion in 3D co-culture model

A study performed by Gao and colleagues, observed that in co-culture model with Jeg-3 spheroids on decidualized stromal cells, BPA treatment (10 μM) suppressed invasion and outgrowth of spheroid (Gao et al., 2019). To evaluate the effect of a BPA (1 pM) pre-exposed endometrium on the HTR-8/SVneo cell migration, we used a direct 3D co-culture model cultured for 48 h. Spheroid outgrowth area was defined by a yellow line. BPA reduced significantly the SPHD outgrowth area onto decidualized tHESCs respect to the control group (*p<0.05). The fluorescent trophoblast labeling confirms the position of cells into 3D co-cultures in each condition and time (24h and 48h) (**Figure 9**).

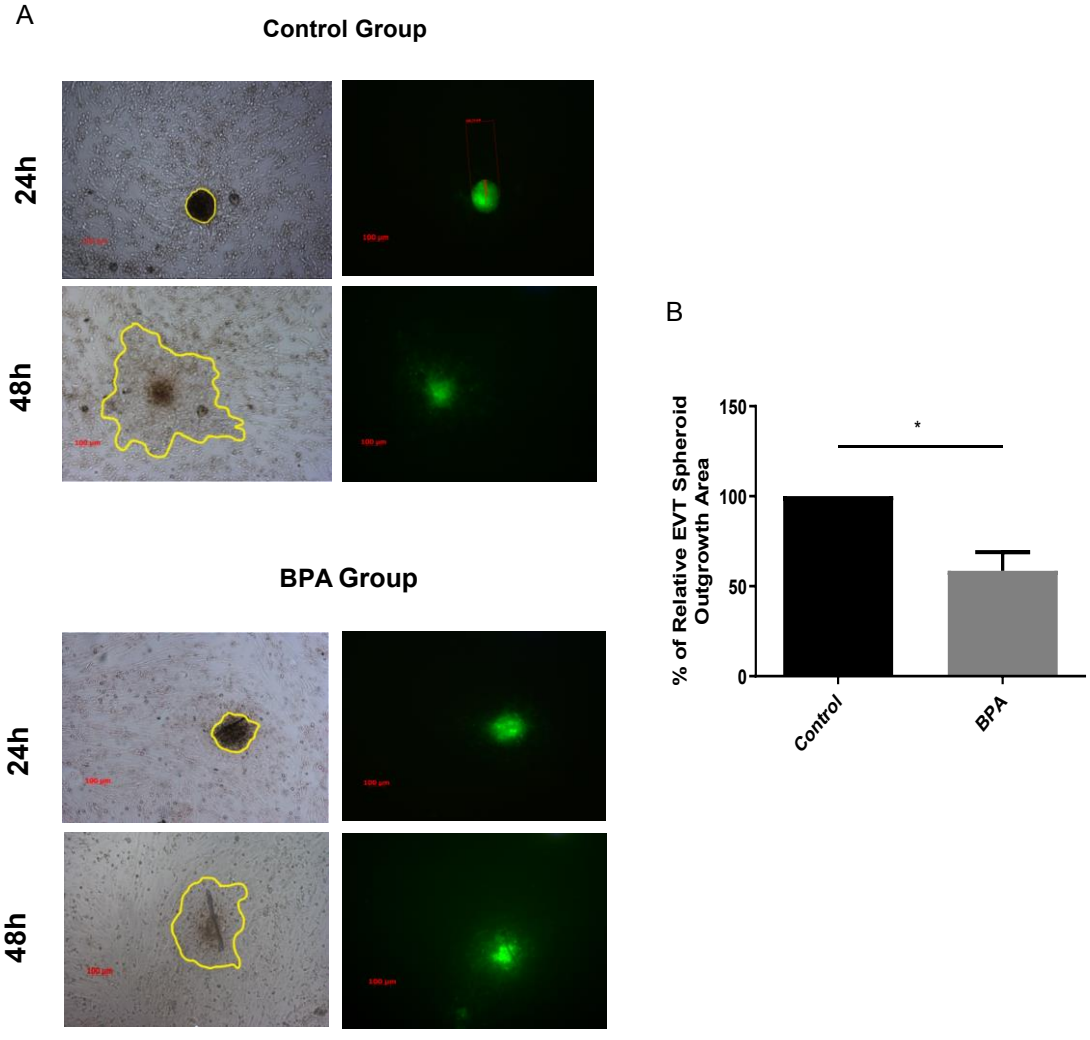

Figure 9. BPA impact on spheroid expansion in 3D co-culture model. (A) Representative images of spheroid outgrowth at 24h and 48h co-cultured with tHESCs pre-treated with 1 pM BPA or with vehicle (Control). Spheroid outgrowth area was defined by a yellow line. (B) Percentual of outgrowth area of BPA group *vs* control. Data are presented as mean ± SE (*p < 0.05) (n = 3).

4.2. Effect of *p*-NP on spheroid expansion in 3D co-culture model

Once demonstrated the effect of BPA on SPHD expansion, the results stimulated us to investigate the effect of *p*-NP on SPHD outgrowth at the same culture conditions. The tHESCs were pre-exposed a two different doses of *p*-NP, 1 nM and 1 pM. Both *p*-NP doses showed a significant reduction in the outgrowth area of SPHD co-cultured with tHESCs previously exposed to the chemical during decidualization respect to control group (*p<0.05 1 pM *vs* control; **p<0.01 1nM *vs* control). (**Figure 10**).

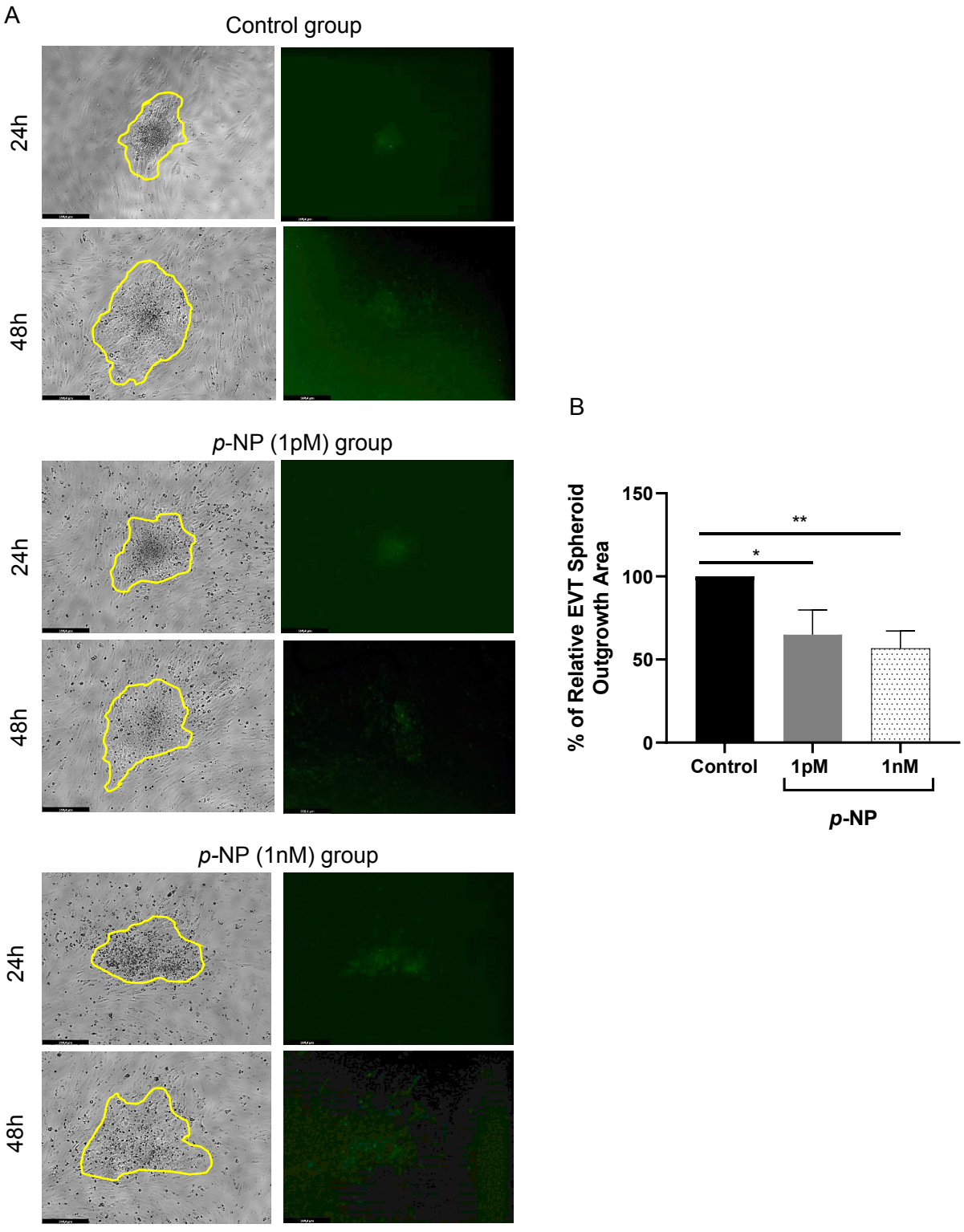

Figure 10. *p*-NP impact on spheroid expansion in 3D co-culture model. (A) Representative images of spheroid outgrowth at 24h and 48h co-cultured with tHESCs treated with 1 pM and 1 nM *p*-NP or only the vehicle (Control). Spheroid outgrowth area was defined by a yellow line. (B) Percentual of outgrowth area of *p*-NP groups *vs* control. Bar scale = 358.4 µm. Data are presented as mean ± SE (*p < 0.05; **p<0.01) (n = 3).

4.3. Effect of *p*-NP during tHESCs decidualization.

In order to elucidate possible factors that are involved in reducing trophoblast migration in the co-culture model, we decided to evaluate the effects on each cell type separately. First of all, was evaluated the effect of *p*-NP during tHESCs decidualization by analysis of two proteins involved in decidualization process, PRL and Connexin 43 (CX-43). The results demonstrated the PRL levels significantly decreased in tHESCs treated with 1 nM and 1 pM of *p*-NP respect to the control group. (**p<0.005; ***p<0.001) (**Figure 11A**). Besides that, the PRL mRNA expression showed a trend of reduction in the cells treated with *p-NP* doses **Figure 11B**). Even though the PRL results demonstrated a decrease in tHESCs decidualization treated with *p*-NP doses, no effect was observed on the CX-43 levels on these cells (**Figure 11C**).

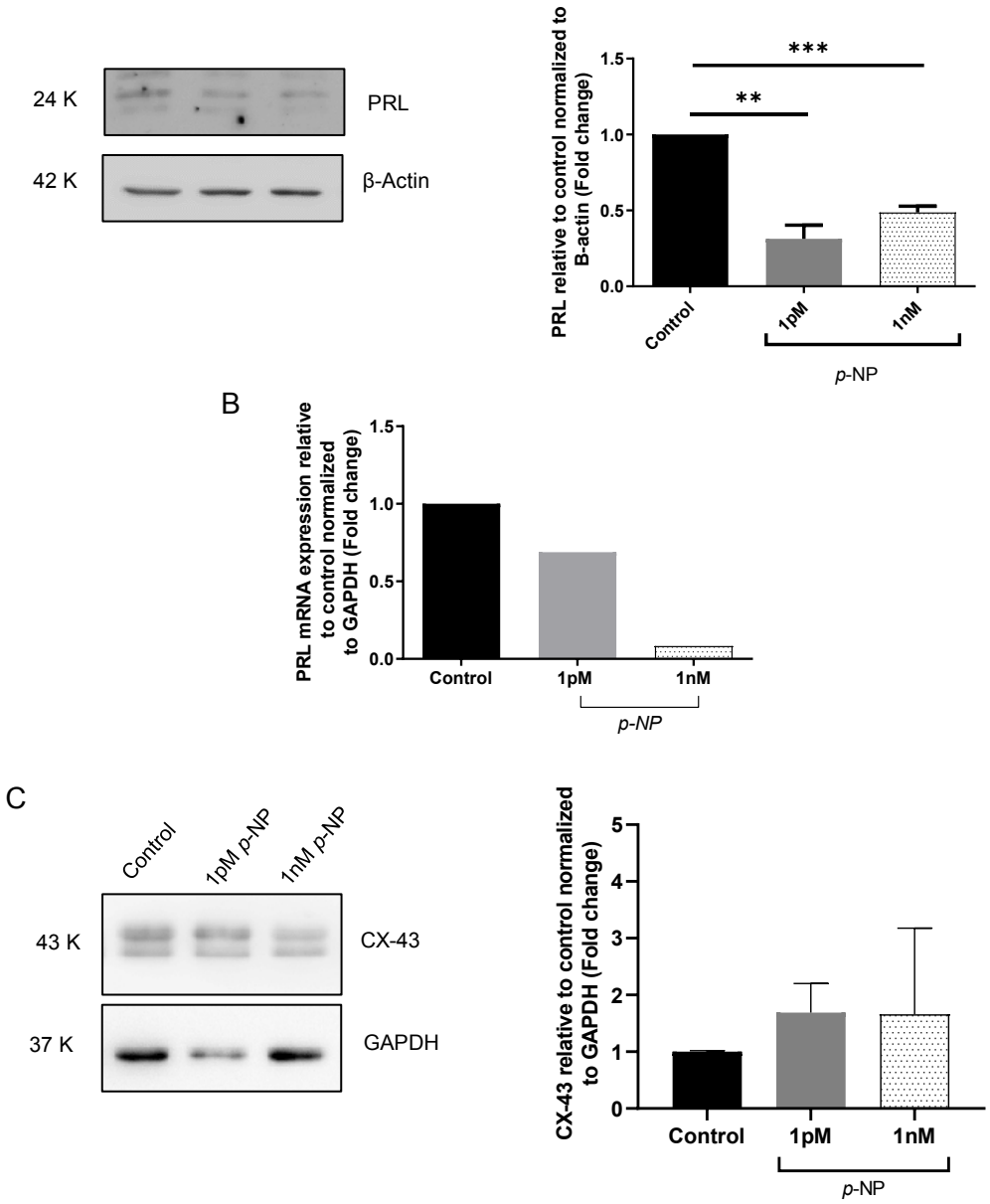

Figure 11. Effect of *p*-NP on prolactin (PRL) and connexin 43 (CX-43) protein level and expression in decidualizing tHESCs treated or not with *p*-NP (1pM and 1nM). (A) Representative WB (left panel) and corresponding densitometry (right panel) of PRL levels in tHESCs treated with *p*-NP (1pM and 1nM) or only the vehicle (Control) (n=3). (B) Fold change of PRL mRNA expression (n=1). (C) Representative WB and densitometry of CX-43 levels (n=3). Data are presented as mean ± SE (**p<0.005; ***p<0.001).

4.4. Migration assay of HTR-8/SVneo cells treated with tHESC conditioned media pre-exposed to *p-NP*

To assess the influence of the conditioned media of tHESC (tHESC-CM), pre-exposed or not to *p*-NP (*p*-NP tHESC-CM), on the migration of HTR-8/SVneo, trophoblast cells were treated with the conditioned media and a scratch migration test was performed. **Figure 12** shows that tHESC-CM reduce significantly the migration of HTR-8/SVneo cells after 18h respect to those cells non treated with tHESC-CM (*p<0.05). Moreover, the *p*-NP tHESC-CM obtained by 1 pM of *p*-NP demonstrated a significant decrease on HTR-8/SVneo migration compared with tHESC-CM of the control group (*p<0.05) (**Figure 13**).

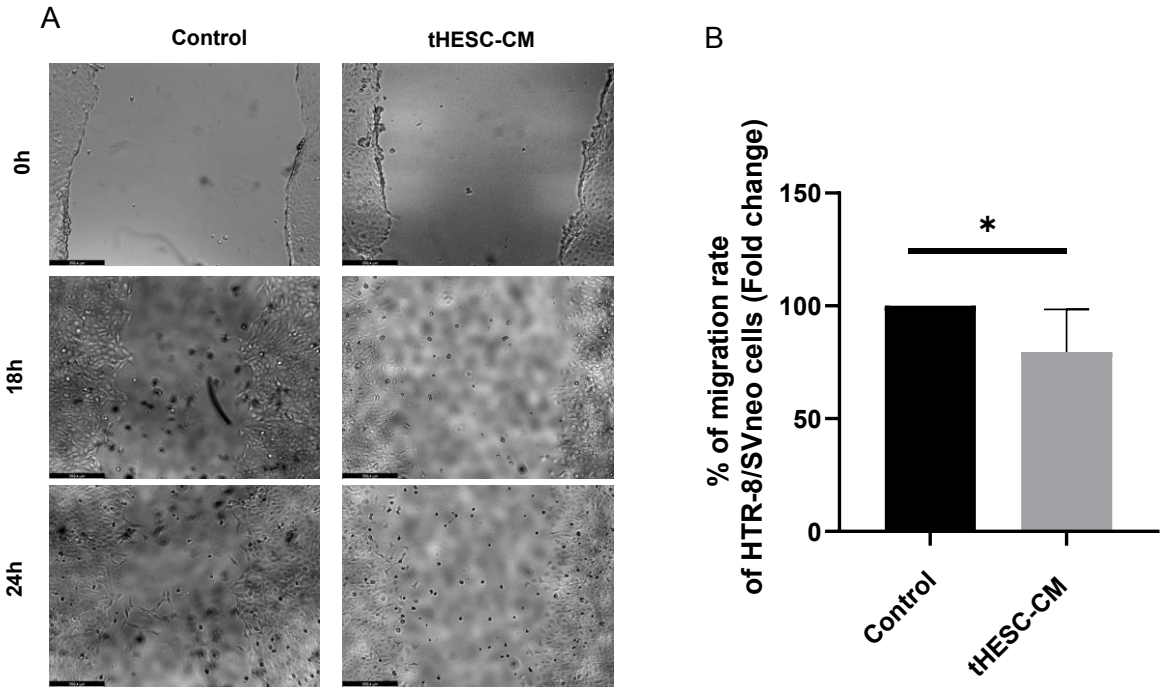

Figure 12. Scratch test. (A) Representative images of HTR-8/SVneo migration treated or not (control group) with tHESC conditioned medium at 0h, 18h and 24h after scratch. (B) Fold change of migration of HTR-8/SVneo. Bar scale = 358.4 µm. Data are presented as mean ± SE (*p < 0.05) (n=3).

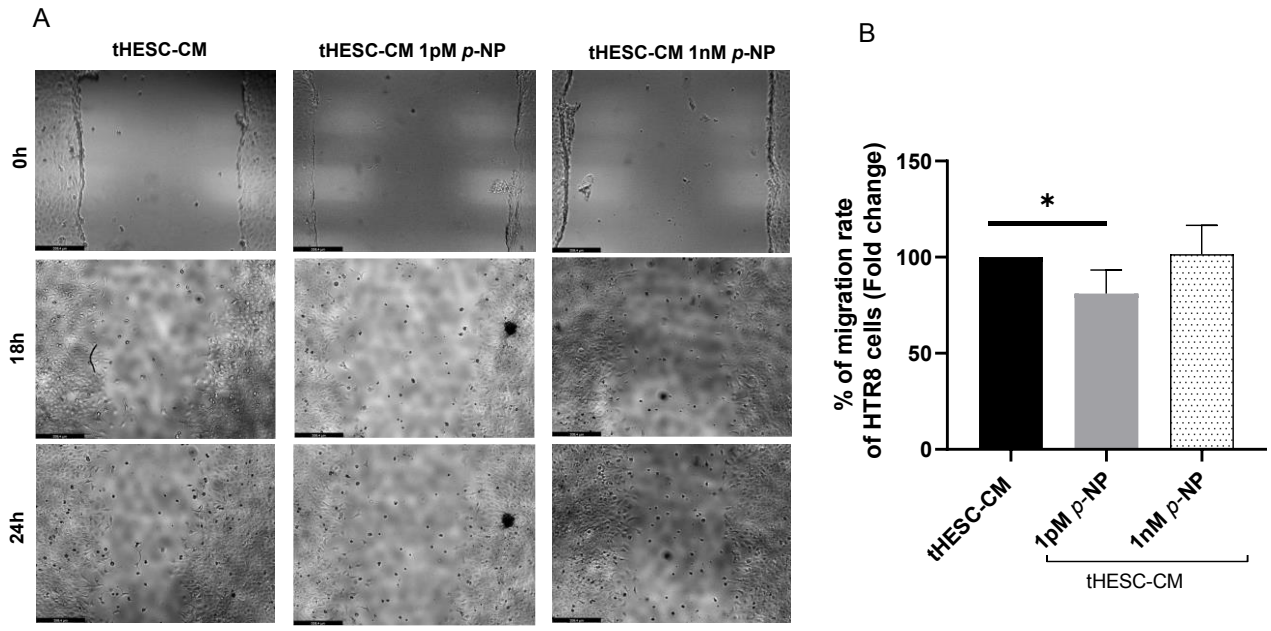

Figure 13. Scratch test. (A) Representative images of HTR-8/SVneo migration with tHESC conditioned medium treated or not (control group) with *p*-NP (1pM and 1nM) at 0h, 18h and 24h after scratch. (B) Fold change of migration of HTR-8/SVneo. Bar scale = 358.4 µm. Data are presented as mean ± SE (*p < 0.05) (n=3).

4.5. The effect of tHESC conditioned media pre exposed to *p*-NP in adhesion of HTR-8/SVneo cells

To better understand how the conditioned media of tHESC pre exposed to *p*-NP interferes with the migration of HTR-8/SVneo cells, the expression of adhesion molecules, such as α5β1 Integrin and CD9 in the trophoblast line, was evaluated. The tHESC-CM obtained by the treatment with *p*-NP had no effect on α5β1 Integrin levels on HTR-8/SVneo at any *p*-NP concentration used while it significantly reduced the CD9 levels at both doses (1pM and 1nM) as compared with the tHESC-CM of control group (*p<0.05; **p<0.01) (**Figure 14 A, B**).

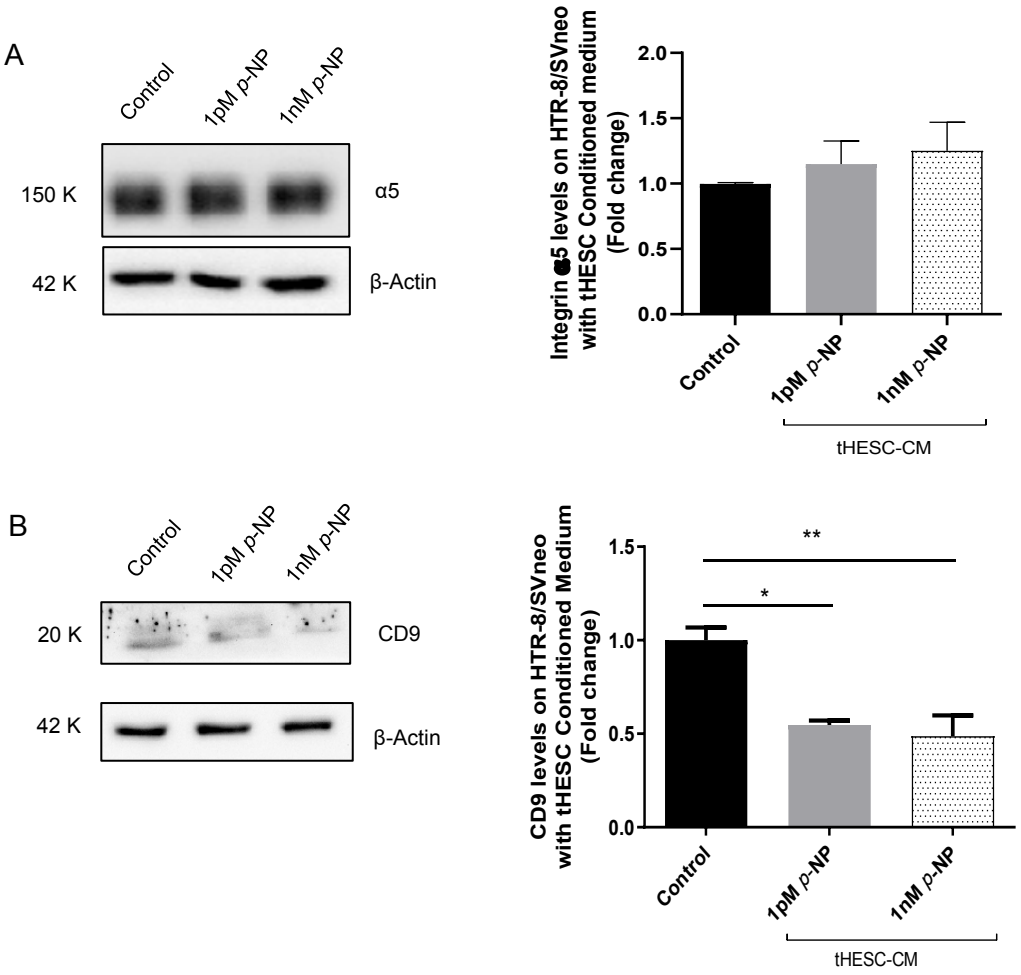

Figure 14. Effect of *p*-NP on Integrin α5 subunit (α5) and CD9 protein levels in HTR-8/SVneo treated with tHESC conditioned medium exposed or not to (Control) *p*-NP (1pM and 1nM). (A) Representative WB (left panel) and corresponding densitometry (right panel) of integrin α5 levels on HTR-8/SVneo (n=3). (B) Representative WB (left panel) and densitometry (right panel) of CD9 levels (n=3). Data are presented as mean ± SE (*p<0.05; **p<0.01).

4.6 Effect of *p*-NP treated tHESC conditioned medium on metalloproteinases in HTR-8/SVneo.

To evaluate the impact of conditioned media from *p*-NP treated tHESCs (tHESC-CM) on trophoblast migration, MMP-9 and MMP-2 levels on HTR-8/SVneo cells were tested. **Figure 15** shows that *p*-NP tHESC-CM induced a reduction in MMP-9, even if not significant compared to tHESC-CM of control group (**Figure 15A**). There was no effect of *p*-NP tHESC-CM on MMP-2 levels in trophoblast cells (**Figure 15B**).

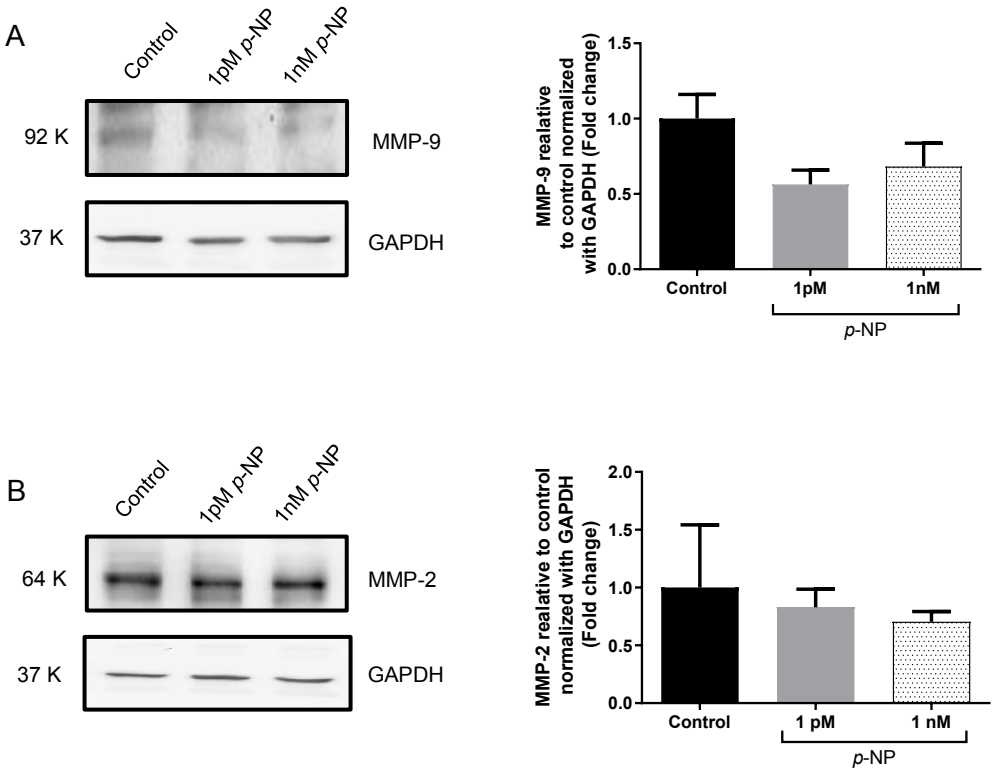

Figure 15. Effect of *p*-NP on metalloproteinases (MMP-9 and MMP-2) levels in HTR-8/SVneo treated with tHESC conditioned medium obtained or not (Control) by treatment with *p*-NP (1pM and 1nM). (A) Representative WB (left panel) and corresponding densitometry (right panel) of MMP-9 levels on HTR-8/SVneo (n=3). (B) Representative WB (left panel) and densitometry (right panel) of MMP-2 levels (n=3). Data are presented as mean ± SE.

4.7. Effect of *p*-NP on lysosome biogenesis during decidualization process.

The mechanism of lysosomal biogenesis is coordinated by the transcription factor EB (TFEB), which regulates the expression of proteins such as LAMP-1 and cathepsin D (CTSD) (Settembre et al 2011). In order to assess the lysosome biogenesis in tHESC decidualized in presence of the chemical, a qPCR was performed to examine the TFEB mRNA expression. tHESCs decidualized in presence of *p*-NP presented a significant increase in TFEB mRNA expression respect to tHESC-CM of control group (*p<0.05) (**Figure 16A**). To further investigate the *p*-NP stimulated biogenesis of lysosomes, a Western blot analysis to examine LAMP 1 protein levels was performed. Treatment with *p*-NP induced an increase even if not significant of LAMP 1 protein levels in decidualized tHESC treated with 1pM of *p*-NP (**Figure 16B**). We then confirmed the rise of lysosome number by qualitative immunofluorescence for LAMP- 1 (red) in the decidualized tHESC treated or not with the chemical. **Figure 16C** shows a higher presence of lysosomes confirming an increase of lysosomes biogenesis on tHESCs exposed to 1pM of *p*-NP as respect to the control group.

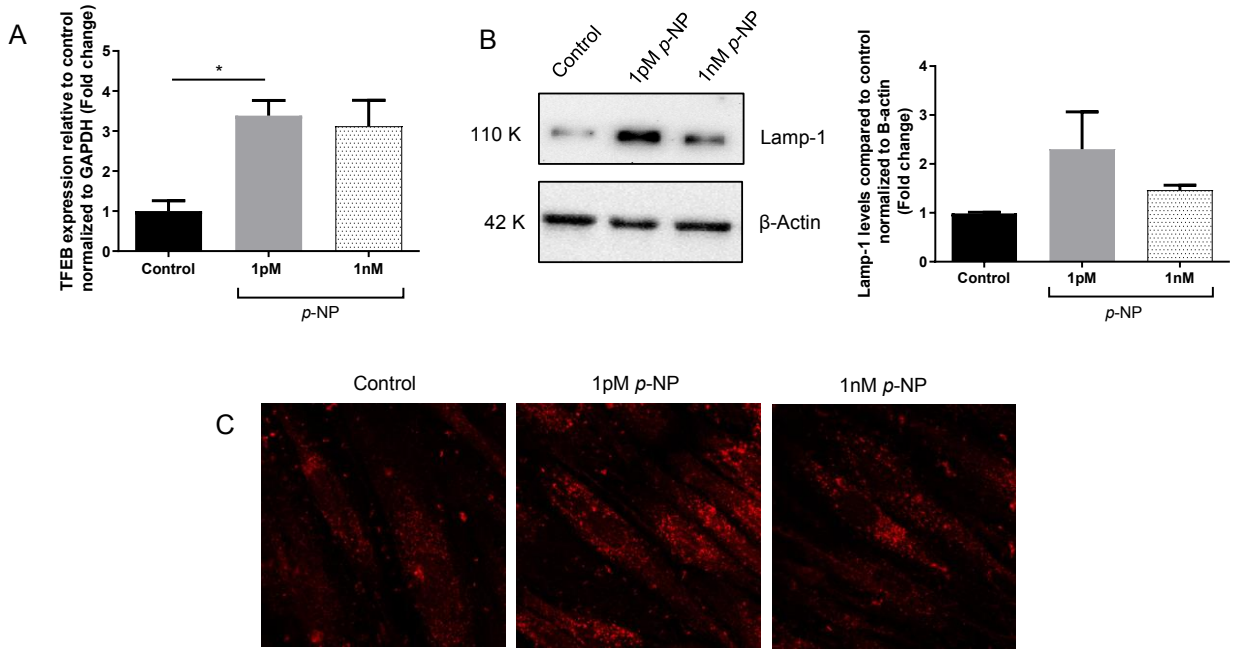

Figure 16. Effect of *p*-NP on TFEB expression and LAMP-1 levels in decidualizing tHESCs treated with *p*-NP (1pM and 1nM) or not (Control). (A) Fold change of TFEB mRNA expression in decidualized tHESC (n=3). Data are presented as mean ± SE (*p<0.05). (B) Representative WB (left panel) and corresponding densitometry (right panel) of LAMP-1 levels during tHESC decidualization (n=3). (C) Qualitative immunofluorescence of LAMP-1 levels in decidualizing tHESCs (n=3). Magnification 40 x .

4.8. Effect of *p*-NP on lysosome exocytosis and extracellular matrix remodeling during decidualization process.

Based on the rise of lysosome biogenesis, we decided to evaluate if also the lysosomal exocytosis was altered by the treatment of the chemical during decidualization. Acid phosphatase is one of the acid hydrolases that are normally present in lysosomes and can be released in the extracellular environment by lysosomal exocytosis, thus, the acid phosphatase activity in the conditioned media was tested. tHESC exposed to 1pM *p*-NP showed a significant decrease in the acid phosphatase activity (*p<0.05 *vs* Control) and those cells treated with 1nM *p*-NP increased significantly the enzyme activity at the conditioned media compared to the tHESC control group (***p<0.001) (**Figure 17 B**).

This data was further corroborated when it was evaluated the intracellular Cathepsin D (CTPS D) protein levels that were significantly decreased on tHESCs treated with 1nM *p*-NP respect to the 1pM *p*-NP group (*p<0.05) (**Figure 17C**). Interestingly, The CTPS D levels on conditioned media increased on tHESCs exposed a 1nM of *p*-NP (**Figure 17 D**).

Based on the fact that CTPS D is capable of degrading extracellular matrix components, we decided to evaluate the intra and extracellular expression of fibronectin. **Figure 18 A** shows that the conditioned media of tHESC exposed to 1nM of *p*-NP presented a significant reduction of fibronectin expression when compared to control group (*p<0.05). On the other hand, intracellular fibronectin expression showed no difference in intracellular fibronectin levels between *p*-NP treated and control groups. (**Figure 18 B**).

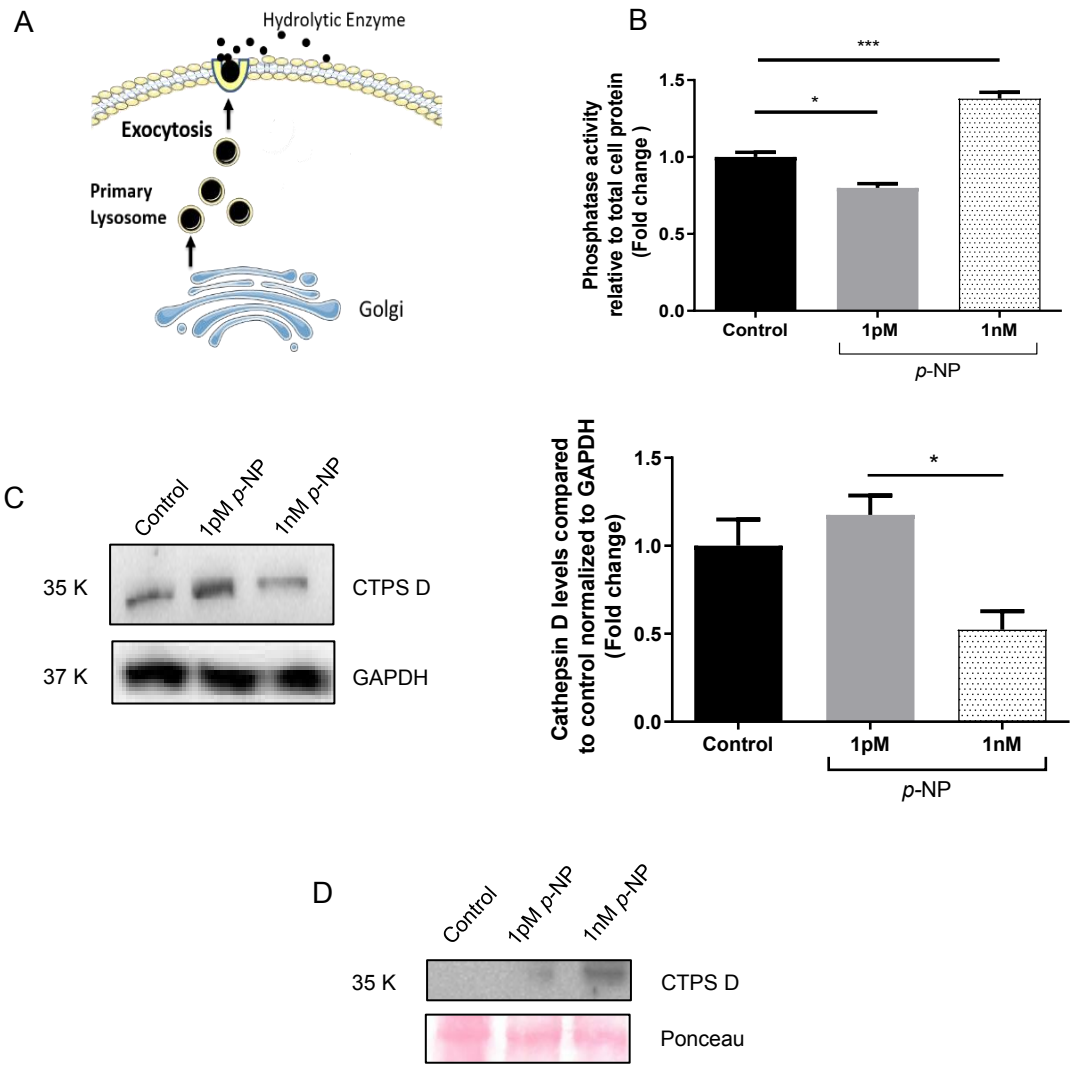

Figure 17. Effect of *p*-NP on acid phosphatase activity and Cathepsin D levels in decidualizing tHESCs exposed or not (Control) to *p*-NP (1pM and 1nM). (A) Image report exocytosis process of hydrolytic enzymes. (B) Acid phosphatase activity on decidualizing tHESCs conditioned medium (n=3). (C) Representative WB (left panel) and corresponding densitometry (right panel) of intracellular Cathepsin D levels during tHESC decidualization (n=3). (D) Representative WB of Cathepsin D levels on decidualizing tHESCs conditioned medium (n=2). Data are presented as mean ± SE (*p<0.05; ***p<0.001).

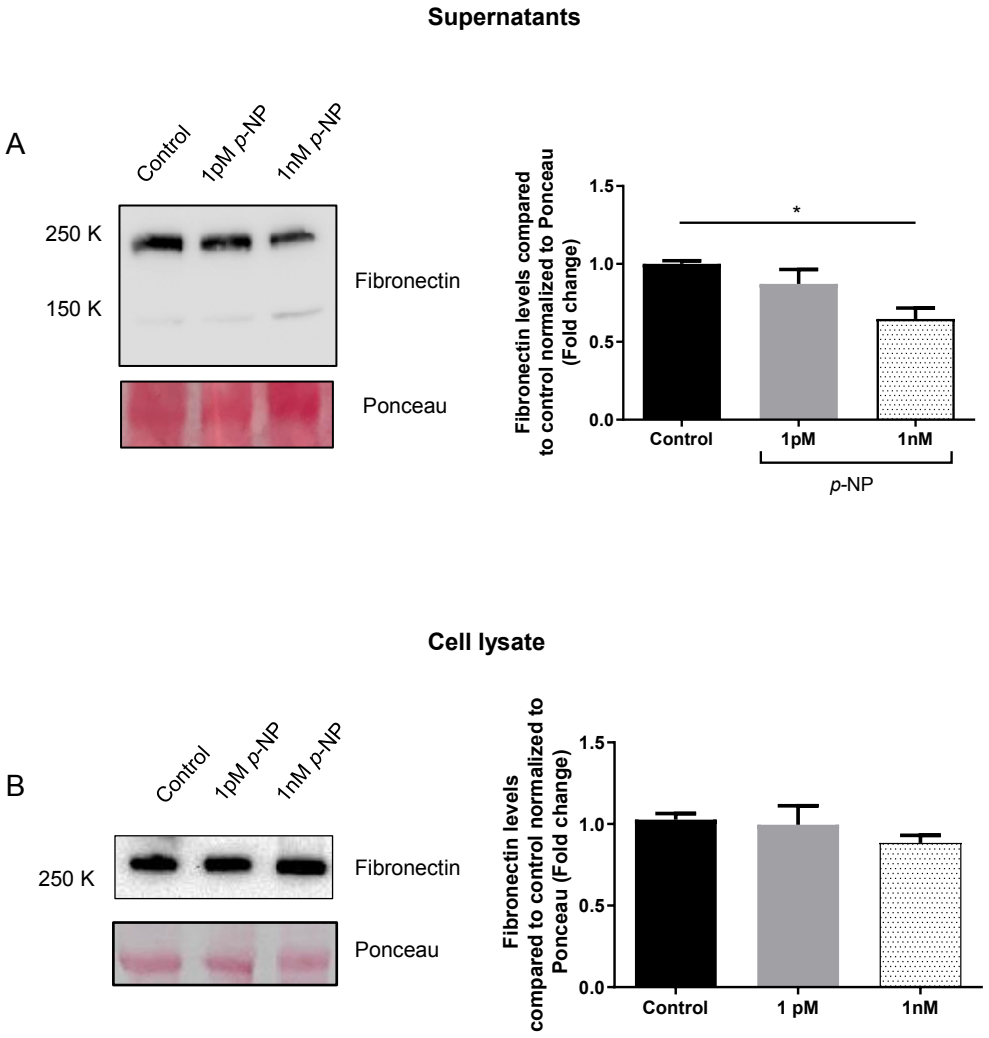

Figure 18. Effect of *p*-NP on Fibronectin levels on decidualizing tHESCs exposed or not (Control) to *p*-NP (1pM and 1nM). (A) Representative WB (left panel) and corresponding densitometry (right panel) of extracellular Fibronectin levels during tHESC decidualization (n=3). (B) Representative WB (left panel) and densitometry (right panel) intracellular of Fibronectin levels on decidualizing tHESCs conditioned medium (n=3). Data are presented as mean ± SE (*p<0.05).

5. Discussion

5.1 Previous studies on the effects of BPA and *p*-NP in cells and tissues at the materno-fetal interface

In 2010, Mork in collaboration with our research group demonstrated that BPA can cross through the placental barrier and reach the fetus (Mørck et al., 2010). In 2011, similarly, Balakrishnan and colleagues showed that *p*-NP can be transferred across the human placenta (Balakrishnan et al., 2011). Due to the capacity of these EDCs to bind to estrogen receptors and regulate several processes in the materno-fetal interface, numerous studies have been dedicated to understanding their effects during pregnancy (Bechi et al., 2006; Mannelli et al., 2015; Paulesu et al., 2018).

Along the years our research group and collaborators have studied the effects of BPA in different aspects inside the materno-fetal interface, such as the interference on decidualization markers (Mannelli et al., 2015), the capacity to increase β-hCG secretion and cell fusion in trophoblast cells (Narciso et al., 2019), and the release of a pro-inflammatory cytokine the macrophage migration inhibitory factor (MIF) by human placenta (Mannelli et al., 2014). Moreover, the research group demonstrated the potential of BPA to increase the expression and plasma membrane location of glucose transporter 1 (GLUT1) (Benincasa et al., 2020) and to reduce GLUT1 levels in the placenta from mothers with metabolic dysfunction (Ermini et al., 2021). Studies on HTR-8/SVneo cells also reported that BPA affects cell invasion by increasing the differentiation process of trophoblast into giant cells (Spagnoletti et al., 2015). In addition, p-NP produces changes in β-hCG secretion and alters the secretion of cytokines (Bechi et al., 2010, 2006).

Several other evidence showed the effect of BPA on trophoblast migration and invasion process in co-culture models with HESCs (Fan et al., 2020a; Li et al., 2018; Wang et al., 2015). In particular, BPA treatment (10 μM) on decidualized stromal cells, suppressed Jeg-3 spheroid invasion and outgrowth in a co-culture model (Gao et al., 2019).

5.2 Our studies on the effect of BPA and *p*-NP on spheroid expansion in 3D co-culture model

Accordingly to previous studies, a direct 3D co-culture model was established during my PhD course, to examine the effect of BPA and *p*-NP at the materno-fetal interface. In this model, trophoblasts and endometrial cells remain in straight contact with each order and allow a direct and instantaneous cross-talk between both types of cells (Manzan-Martins and Paulesu, 2021). In order to better understand the effects of BPA and *p*-NP on pregnancy, the doses of these chemicals tested in our studies corresponded to those normally found in human tissues (1nM) or even lower (1 pM).

The studies on BPA demonstrated that even in HTR-8/SVneo cell line the outgrowth area of spheroids is reduced when these cells are in co-culture with decidualizing tHESCs, pre-treated with a low dose of BPA (1 nM).

We then decided to start a long study on *p*-NP as the effects of this chemical are still largely unknown. Similarly to BPA, *p*-NP reduced the HTR-8/SVneo spheroid outgrowth area when these cells were in contact with decidualizing tHESCs pre-exposed to 1pM and 1nM *p*-NP. The nontoxicity of used concentrations of *p*-NP was previous demonstrated by Bechi and colleagues (Bechi et al., 2013).

These results demonstrated that contamination of the endometrium with EDCs such as BPA and *p*-NP, in a fertile, non-pregnant woman, can cause changes in the endometrial cells which could be reflected in an alteration of the cross talk of these cells with trophoblast, in the event of a pregnancy.

5.3 Effect of *p*-NP on tHESCs decidualization

It is well known that processes of decidualization and remodeling of the extracellular matrix play a critical role in the invasion process of trophoblast and the endometrium (Gellersen et al., 2007; Olson et al., 2017). Wongwananuruk and collaborators (2016), reported that in a co-culture model, decidualizing endometrial stromal cells were able to regulate the HTR-8/SVneo spheroid expansion and invasion (Wongwananuruk et al., 2016). Therefore we decided to verify the effects of *p*-NP on such processes.

The decidualization process is characterized by morphological and biochemical transformations of the endometrium and is essential for implantation of the embryo (Gellersen et al., 2007; Olson et al., 2017). The literature reported that EDCs can alter the endometrium decidualization changing the expression of decidualization markers (Aghajanova and Giudice, 2011; Xiong et al., 2020). Prolactin (PRL) is one of decidualization markers and starts to be detected within 24h (Pan-Castillo et al., 2018). Our results showed that exposition of tHESCs to *p*-NP decreases cell decidualization reducing the levels of PRL marker.

Connexins are a family of integral membrane proteins that are involved in the formation of gap junctions. These proteins were detected in first-trimester villous and extravillous trophoblast and showed to be involved in human trophoblast differentiation and cell fusion

(Frendo et al., 2003; Kibschull et al., 2008; Winterhager et al., 1999). The gap junction formation, mediated by connexin 43 (CX-43) increases in the decidualization process, and can maintain the connection between cells, limiting the trophoblast invasion and migration. For this reason, CX-43 is used as a biomarker of decidualization (Wongwananuruk et al., 2016). Interestingly, in endometriotic conditions, where the trophoblast invasion is increased, there is a reduction on CX-43 levels on cultured endometrial stromal cells (Yu et al., 2014). In our study, the tHESC exposition to *p*-NP presented no changes in levels of CX-43. Therefore, although *p*-NP impairs decidualization of tHESC, as demonstrated by the decrease of PRL, the lack of action on the GAP junctions suggests that, under the effect of *p*-NP, endometrial cells retain, at least in part, the ability to limit the migration and invasion of trophoblast cells.

5.4 Effect of *p*-NP on HTR-8/SVneo cell migration.

The 3D co-culture model showed a reduction in HTR-8/SVneo spheroid migration placed into decidualizing tHESC monolayer exposed to *p*-NP. This suggested that *p*-NP exposure produces changes in the endometrial stromal cells triggering the release of signals that are able to control the migration and invasion of trophoblast process. To better understand this complex cross-talk between trophoblast and endometrial stromal cells we decided to perform experiments on an indirect co-culture model in which, tHESC conditioned medium was tested on the migration of HTR-8/SVneo cells, using a scratch test. Our findings showed a decrease in trophoblast migration confirming that secretory factors from decidualizing stromal cells can regulate trophoblast migration.

Interestingly, Menkhorst and co-workers showed that the conditioned medium from endometrial cells with incomplete decidualization had a higher effect than that from complete decidualization (2 days *versus* 14 days of decidualization) (Menkhorst et al., 2019). On these bases, we performed studies on *p*-NP using conditioned media from tHESCs at incomplete decidualization and showed that the capacity of tHESC conditioned media in reducing HTR-8/SVneo cell migration was enhanced by pre-exposition of tHESCs to *p*-NP. Importantly, the effect of *p*-NP was higher at a lower concentrations (1pM). Altogether this data indicate that an altered decidualization of tHESCs induced by low doses of *p*-NP impairs the cross-talk between trophoblast and endometrial stromal cells.

5.5 Effect of *p*-NP on trophoblast adhesion molecules

During trophoblast migration process, significant interactions are formed between cells and extracellular matrix (ECM). The adhesion molecules play a crucial role in this process in which cell surface integrins interact with fibronectin and collagen, the important components of ECM. In literature was reported that during trophoblast invasion and migration the fibronectin binding integrin α5β1 expression is upregulated (Damsky et al., 1994). We evaluated the α5 integrin levels on HTR-8/SVneo treated with conditioned media from tHESC-CM pre-exposed to *p*-NP and found that there was no difference in this integrin level compared to the control. Thus, the *p*-NP did not impair the adhesion of trophoblast to fibronectin by α5β1 integrin.

However, it has been demonstrated that the CD9 molecule, a cell surface glycoprotein, is present in extravillous trophoblast (EVT) and was associated with α5 integrin (Fujiwara et al., 2018; Hirano et al., 1999a). CD9 was related to the invasive characteristics of BeWo cells

mediated by α5β1 integrin and its interaction with fibronectin facilitating the invasion and migration of trophoblast (Hirano et al., 1999b). As we observed in our study, there was a significant decrease in CD9 levels on HTR-8/SVneo treated with tHESC-CM exposed to *p*-NP. So the reduction in trophoblast migration could be connected to the decreased levels of CD9 and therefore to a reduced interaction between cells and components of ECM at the materno–fetal interface.

5.6 Effect *p*-NP on metalloproteinases.

Another explanation for the effect of *p*-NP on reduction of trophoblast migration could reside in the MMPs modulation by *p*-NP. During placental development, trophoblast secretes metalloproteinases that can degrade and remodel the ECM, facilitating the invasion and migration of EVTs (Graham and Lala, 1991). Many studies have reported that alterations on MMP-2 and MMP-9 expressions or the balance MMP/TIMP are correlated with changes in invasion and migration in the decidua (Wang et al., 2015; Ye et al., 2019). Even more, was demonstrated that EDCs exposition reduces the migration process in HTR-8/SVneo cells, by downregulation of MMP-2/9 and by decreasing the integrin α5β1 and vimentin expression (Wei et al., 2020). On this work, HTR-8/SVneo in contact with conditioned media from tHESC exposed to *p*-NP presented a reduced protein expression of MMP-9, even if not statistically significant.

We can therefore speculate that alteration of decidualization induced by *p*-NP, leads to altered communication between stromal and trophoblast cells that affects the migration process by downregulation of CD9 and MMP-9 protein levels.

Success of pregnancy requires correct embryo development, endometrial decidualization, and placenta formation. Any disturbance in the processes of decidualization could lead to embryo implantations failure, miscarriage or disorders on placentation and/or intrauterine fetal growth restriction (Cha et al., 2012; Gellersen et al., 2007).

5.7 Effect of *p*-NP on lysosome biogenesis and exocytosis during decidualization process.

Successful embryo implantation is associated with dynamic changes in the endometrium (Ye, 2020). Recent studies indicated that changes at the implantation site were induced entirely by the enzymatic alterations in the endometrium itself. Dynamic presence of lysosomes and activities of lysosomal enzymes were detected in the endometrium leading to the establishment of a receptive uterus (Wood, 1973). Studies on mouse model showed that increased number of lysosomes and lysosomal enzymes from non-decidualized stromal cells to mature decidual cells suggests the involvement of lysosomes in the differentiation of endometrial stromal cells into decidual cells to accommodate embryo development and placental development (Bijovsky and Abrahamsohn, 1992).

The transcription factor EB (TFEB) is responsible to coordinate lysosome biogenesis, by controlling the expression of lysosomal associated membrane protein 1 and 2 (LAMP-1 and LAMP-2), as well as the hydrolytic enzymes present in the acidic organelle (Medina et al., 2011; Settembre et al., 2011). We observed that tHESCs treated with *p*-NP exhibited an increase of TFEB expression compared to non-exposed ones, suggesting that the chemical could trigger the increase of lysosomal biogenesis.

To confirm the increase of lysosome biogenesis, we measured the levels of LAMP-1 protein and we observed an increase even if no statistically significant, on the tHESCs treated with *p*-NP. Moreover, we observed not only a rise in the number of lysosomes, but also their targeting to the plasma membrane suggesting an increasing in lysosomal exocytosis.

In order to elucidate the increase of lysosomal exocytosis we have measured the activity of lysosomal hydrolytic enzyme acid phosphatase in the conditioned media of the tHESC.

During exocytosis , these enzymes are secreted into the extracellular medium and can be measured by a period of time (Rodríguez et al., 1997; Samie and Xu, 2014). We observed an increase of acid phosphatase activity in the conditioned media of tHESCs exposed to *p*-NP further confirming the lysosomal exocytosis observing. The lysosomal exocytosis releases a variety of hydrolases such as, phosphatases, proteases, lipases, carbohydrases, sulphatases (Kolter and Sandhoff, 2005; Schulze and Sandhoff, 2011) in the extracellular environment. During pregnancy Cathepsins regulate well-defined events of trophoblast invasion migration, adhesion by remodeling the ECM (Ishida et al., 2004). Our data showed a decrease on intracellular levels of Cathepsin D and an increase of extracellular levels, demonstrating that treatment with 1nM of *p*-NP stimulate the release of Cathepsin D by tHESC. During the initial phases of placentation Cathepsins degrade components of ECM to facilitate the trophoblast invasion, but after 2 weeks of pregnancy decidualized endometrial secrete cystatin C, one of the main Cathepsin inhibitor (Laurent-Matha et al., 2012). Furthermore, it was reported that a raise in cathepsins levels is associated with recurrent spontaneous miscarriages and the deregulation on its proteolytic activity may lead a gestational miscarriages (Nakanishi et al., 2005).

It is well reported in literature that extracellular cathepsins present in the ECM are responsible for a controlled degradation of fibronectin, collagen and elastin (Buck et al., 1992; Sloane and Honn, 1984). Our study showed a significant decrease of extracellular fibronectin in tHESCs exposed to *p*-NP, while the intracellular protein levels did not change, so we can speculate that the fibronectin was degraded in the extracellular compartment by the Cathepsin D released in the extracellular media.

Thus, the *p*-NP impaired decidualization by lead to an increase of lysosomal exocytosis with a consequently cathepsin released. The reduction of extracellular fibronectin can be associated with the reduction of trophoblast migration. In fact, fibronectin acts as a bridging ligand mediating adhesion and, then, migratory activity of trophoblasts cells by the interaction with α5β1 integrin receptor and CD9 (Ilić et al., 2004).

6. Conclusive Remarks

In conclusion, this study showed that pre-exposition of endometrial stromal cells to endocrine disruptors, bisphenol A and *para*-nonylphenol, leads to an impaired decidualization, as well as lysosomes biogenesis and trophoblast cell migration. These changes might compromise the implantation of the placenta and its development leading to complications during pregnancy or even gestational loss. Our data encourage to investigate the possible pathways involved in this important and complex cross-talk between uterus and placenta, at the materno-fetal interface.

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